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(72) Inventors: **BUCK, Jochen**; 4 Ferris Drive, Old Greenwich, CT 06870 (US). **LEVIN, Lonny, R.**; Apt. 6D, 1161 York Avenue, New York, NY 10021 (US).

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(74) Agents: **SEMIONOW, Raina** et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022-7513 (US).

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(71) Applicant: **CORNELL RESEARCH FOUNDATION, INC.** [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US).

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(54) Title: MAMMALIAN SOLUBLE ADENYLYL CYCLASE

(57) Abstract: The invention provides isolated animal soluble adenylyl cyclase and methods of modulating its expression and activity. Also provided are methods of utilizing soluble adenylyl cyclase for diagnosing pathological conditions and monitoring blood gases.

MAMMALIAN SOLUBLE ADENYLYL CYCLASE

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FIELD OF THE INVENTION

5 The present invention relates to isolated animal soluble adenylyl cyclase and its role in the regulation of the cAMP signaling pathway.

BACKGROUND OF THE INVENTION

Adenylyl cyclase (AC) is the effector molecule of one of the most widely used signal transduction pathways. Its product, cyclic AMP (cAMP), is a nearly
10 universally utilized second messenger molecule, which mediates cellular responses to nutritional conditions and extracellular signals in organisms from prokaryotes to higher eukaryotes. cAMP has long been known to exert both stimulatory and inhibitory effects on cell growth and proliferation (Dumont, J.E., *et al.*, Trends Biochem. Sci., 1989, 14:67-71; Rozengurt, E., Science, 1986, 234:161-6). In metazoans, a seemingly ubiquitous
15 membrane-associated AC activity is encoded by a family of transmembrane adenylyl cyclases (tmACs) that mediate cellular responses to external stimuli.

Throughout the animal kingdom members of the transmembrane adenylyl cyclase (tmAC) superfamily synthesize cAMP to mediate communication between cells (Sunahara, R.K., *et al.*, Annu. Rev. Pharmacol. Toxicol., 1996, 36: 461-80; Taussig, R., *et al.*, J. Biol. Chem., 1995, 270:1-4). For example, in mammals, signals arising from other
20 cells such as hormones, neurotransmitters, and olfactants, modulate tmAC activity via cell surface receptors and G proteins (Taussig, R., *et al.*, Adv. Second Messenger Phosphoprotein Res., 1998, 32:81-98). A similar cAMP signaling cascade is present in other multicellular organisms, including *Drosophila* (Cann, M.J. *et al.*, Adenylyl Cyclases,
25 32. Lippincott-Raven, 1998; Cann, M.J. *et al.*, Adenylyl Cyclase, 32. Lippincott-Raven, 1999; Iourgenko, V., *et al.*, FEBS Lett., 1997, 413:104-8; Iourgenko, V. *et al.*, "A calcium inhibited *Drosophila* adenylyl cyclase (submitted); Levin, L.R., *et al.*, Cell, 1992, 68:479-

89), *C. elegans* (Bargmann, C.I., *et al.*, Science, 1998, 282:2028-33; Berger, A.J., *et al.*, J. Neurosci., 1998, 18:2871-80-; Korswagen, H.C. *et al.*, Embo J., 1998, 17:5059-65), and Dictyostelium (Pitt, G.S. *et al.*, Cell, 1991, 69:305-15). In contrast, the ACs found in unicellular eukaryotes and bacteria transmit nutritional information to the inside of the cell
5 (Danchin, A., Adv. Second Messenger Phosphoprotein Res., 1991, 27:109-62).

Current models for cAMP signal transduction in mammals involve only transmembrane adenylyl cyclases (tmACs), which generate cAMP near the plasma membrane (Hempel, C.M., *et al.*, Nature, 1996, 384:166-9; Sunahara, R.K., *et al.*, Annu. Rev. Pharmacol. Toxicol., 1996, 36:461-80; Taussig, R., *et al.*, J. Biol. Chem., 1995,
10 270:1-4). With the major effector of cAMP, the cAMP-dependent protein kinase (PKA), tethered to intracellular sites often far removed from the plasma membrane by a family of A Kinase Anchoring Proteins (AKAP) (Lester, L.B. *et al.*, Recent Prog. Horm. Res., 1997, 52:409-29; Pawson, T., *et al.*, Science, 1997, 278:2075-80) these models depend upon diffusion of cAMP past membrane-proximal targets to activate intracellular PKA at more
15 distal sites. Furthermore, it must survive in a cytoplasm filled with phosphodiesterases (Beavo, J.A., *et al.*, Mol. Pharmacol., 1994, 46:399-405; Bushnik, T., *et al.*, Biochem. Soc. Trans., 1996, 24:1014-9). However the evidence for cAMP diffusion is based on exogenous addition of millimolar concentrations of cAMP (Bacskai, B.J., *et al.*, Science, 1993, 260:222-6), and experiments which demonstrate diffusion of liberated PKA catalytic
20 subunit (Bacskai, B.J., *et al.*, Science, 1993, 260:222-6; Hempel, C.M., *et al.*, Nature, 1996, 384:166-9). Thus there has not been a satisfactory explanation for the problems associated with how these models operate.

Soluble Adenylyl Cyclase

In addition to tmACs, another type of AC activity has been described in
25 mammals, that of soluble adenylyl cyclase (sAC), which is thought to be expressed only in testis and sperm (Ahn, S., *et al.*, Mol. Cell Biol., 1998, 18:967-77; Bacskai, B.J., *et al.*, Science, 1993, 260:222-6). sAC activity appears to be biochemically and chromatographically different from tmACs, particularly a genetically engineered tmAC which is soluble, and soluble guanylyl cyclases previously described in testis (Neer, E.J., J.
30 Biol. Chem., 1978, 253:5808-5812; Neer, E.J. *et al.*, Biochim. Biophys. Acta, 1979, 583:531-534; Braun, T. *et al.*, Biochim. Biophys. Acta, 1977, 481:227-235). Unlike the

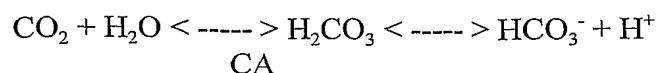
known tmACs, sAC biochemical activity has been shown to depend on the divalent cation Mn 2^+ (Braun, T and Dods, R.F., Proc. Natl. Acad. Sci. USA, 1975, 72:1097-1101), sAC is insensitive to G protein regulation (Braun, T. et al., Biochim. Biophys. Acta, 1977, 481:227-235), and sAC displays approximately 10-fold lower affinity for the substrate ATP (K_m approximately equal to 1 mM) (Neer, E.J., J. Biol. Chem., 1978, 253:5808-5812; Gordeladze, J.O. et al., Mol. Cell Endocrinol., 1981, 23:125-136; Braun T., Methods Enzymol., 1991, 195:130-136) than the tmACs (K_m approximately equal to 100 μ M) (Johnson, R.A. et al., Methods Enzymol., 1994, 238:56-71). Based on these studies, this soluble form of AC was thought to be molecularly distinct from tmACs (Beltran, C. *et al.*, Biochemistry, 1996, 35:7591-8; Berkowitz, L.A., *et al.*, Mol. Cell Biol., 1989, 9:4272-81).

Semipurified soluble adenylyl cyclase activity is inhibited by submicromolar amounts of catechol estrogens (Braun, T., Proc. Soc. Exp. Biol. Med., 1990, 194:58-63). Braun demonstrated that the two hydroxyls of the catechol moiety were essential for the inhibitory interaction, estradiol and estrone were completely inactive. Catechols with aliphatic side chain like dopamine, L-dopa, and norepinephrine were able to inhibit sAC activity, but were 1,000 fold less potent.

Molecular evidence confirming that soluble AC represents a distinct form of adenylyl cyclase is lacking. Thus a need remains for the identification, cloning, characterization and purification of the signaling molecule having soluble adenylyl cyclase activity. There is a further need to modulate sAC activity in order to affect cell function.

Carbon dioxide and bicarbonate

Carbon dioxide (CO_2) is the end product of metabolism in animals. It is normally released into the atmosphere via breathing, but is also soluble in cell membranes. CO_2 combines with water in the presence of carbonic anhydrase (CA) to form carbonic acid (H_2CO_3) which dissociates to liberate a proton and bicarbonate ion (HCO_3^-).



By itself, this reaction reaches equilibrium after about 4 minutes. However, in most biological systems, due to the ubiquitous presence of carbonic anhydrase, bicarbonate/ CO_2 equilibrium is reached nearly instantaneously (Johnson, L.R. Essential Medical

Physiology, 1998, Phila. Lippincott-Raven).

In mammals, blood is a bicarbonate/CO₂ buffer system, and the relationship between blood pH, bicarbonate and CO₂ partial pressure can be described by the Henderson-Hasselbach equation:

5
$$\text{pH} = 6.1 + \log([\text{HCO}_3^-]/0.03P_{\text{CO}_2})$$

This equilibrium in serum is tightly controlled in two ways; the kidneys regulate the bicarbonate concentration and the breathing frequency determines the concentration of carbon dioxide.

Bicarbonate is the carbon source for the initial reactions of gluconeogenesis and ureagenesis (Henry, Annu. Rev. Physiol., 1996, 58:523-538). Additionally, CO₂ and/or bicarbonate have been shown to modulate a number of physiological processes (i.e., diuresis, breathing, blood flow, cerebrospinal fluid formation, aqueous humor formation, and spermatocyte development). In most cases, the effects of CO₂ have been ascribed to as yet undescribed chemoreceptors, and the effects of bicarbonate are usually thought to be mediated by changes in cellular pH (Johnson, 1998).

Measurement of physiological levels of bicarbonate is typically determined indirectly by calculation from the direct measurement of carbon dioxide and pH using the Henderson Hasselbalch equation. However, a certain degree of error is inherent in indirect measurements, for example, due to artifacts in arterial blood sampling. Such errors may have grave consequences to the treatment of acutely ill patients, particularly neonates, and impairs the proper diagnosis of conditions such as respiratory and metabolic acidosis or alkalosis. Indeed, current state-of-the-art portable instruments, useful in emergency or point-of-site testing, such as the i-Stat®, or SenDx 100® only measure pH, CO₂, and PO₂ and calculate the bicarbonate levels based on these measurements. Therefore, there is a need for more accurate and direct determination of physiological bicarbonate levels.

Oncogenesis

In the yeast *Saccharomyces cerevisiae*, orthologs of the mammalian Ras oncogene control the cell's physiological response to nutritional status by modulating adenylyl cyclase activity (Mybonyi, K., *et al*, Mol. Cell Biol., 1990, 10:4518-23; Toda, T.,

et al., Cell, 1985, 40:27-36; Wigler, M., *et al.*, Cold Spring Harb. Symp. Quant. Biol., 1988, 53:649-55). Yeast AC, which is encoded by the *cyr1* gene, is found in a complex with Cyclase Associated Protein (CAP); this association with CAP is required for Ras-responsiveness (Field, J., *et al.*, Cell, 1990, 61:319-27; Gerst, J., *et al.*, Mol. Cell Biol., 5 1991, 11:1248-57). Interestingly, cAMP regulation by Ras proteins in *S. cerevisiae* is the only biochemical pathway in yeast not thought to be conserved in mammals.

In mammals, a broad family of Ras-related, small GTP-binding proteins seems to be involved in cell growth, proliferation, and differentiation (Bos, J.L., *et al.*, EMBO J., 1998, 17:6776-82). Ras genes are potent oncogenes; they are thought to be 10 mutated in 30% of all human tumors. Although the mechanism of Ras transformation in human tumorigenesis has been the focus of intense research over the past years (Chang, E.H., *et al.*, Nature, 1982, 297:479-83; Der, C.J., *et al.*, Proc. Natl. Acad. Sci. USA 79:3637-40; Goldfarb, M., *et al.*, Nature, 1982, 296:404-9; Parada, L.F., *et al.*, Nature, 1982, 297:474-8), its mechanism of oncogenic transformation is still not completely 15 understood. A protein kinase cascade involving Raf protein kinase activation of the MAPKinase cascade is downstream from Ras, as are activation of PI-3 kinase and the Ras-family member Ra1 (Gille, H., *et al.*, J. Biol. Chem., 1999, 274:22033-40; Osada, M., *et al.*, Mol. Cell Biol., 1999, 19:6333-44; Rosario, M., *et al.*, Embo. J., 1999, 18:1270-9). However, these do not tell the entire story; a number of other genes have been proposed to 20 play a role in Ras transformation (Tang, Y., *et al.*, Mol. Cell Biol., 1999, 19:1881-91). Among the best characterized Ras effectors, constitutively active forms of Raf or the various MAPKinase can transform fibroblasts on their own, suggesting this kinase cascade can mediate at least part of the transforming functions of Ras. But oncogenic forms of these kinases are not as efficient at transforming fibroblasts as oncogenic forms of Ras, 25 and inhibition of MAPKinase activity does not completely block transformation of Ras (Denouel-Galy, A., *et al.*, Curr. Biol., 1998, 8:46-55; Yip-Schneider, M.T., *et al.*, Int. J. Oncol., 1999, 15:271-9). In contrast, constitutive activation of PI-3 kinase does not transform cells, but inhibiting its activity is sufficient to prevent transformation by oncogenic Ras (Rodriquez-Viciana, P., *et al.*, Cell, 1997, 89:457-67). Together, these data 30 suggest that signaling through PI-3 kinase is necessary but not sufficient for oncogenic transformation by Ras proteins, while the MAPKinase pathway is at least partially sufficient, but does not appear to be necessary. Thus, there is a need to identify other

components of Ras⁻ mediated transformation.

SUMMARY OF THE INVENTION

The present invention provides soluble adenylyl cyclase (sAC), a signaling enzyme which produces cAMP in eukaryotic, particularly non-yeast eukaryotic cells. The natural soluble form of adenylyl cyclase generates cAMP at a distance from the membrane, and thus closer to its required site of action. Accordingly, soluble adenylyl cyclase is useful in regulating or controlling cAMP production.

In a first aspect, the present invention provides an isolated nucleic acid molecule encoding a soluble adenylyl cyclase. The nucleic acid molecule is selected from the group consisting of a nucleic acid which encodes a polypeptide having an amino acid sequence as set out in SEQ ID NO. 1 or SEQ ID NO: 11, a splice variant thereof or an allelic variant thereof. Alternatively, the invention provides a nucleic acid molecule which hybridizes under stringent conditions to the nucleic acid sequence set out in SEQ ID NO: 2 or SEQ ID NO: 12. A nucleic acid molecule having at least a twenty nucleotides sequence identical to a corresponding twenty nucleotide sequence as set out in SEQ ID NO:2 or SEQ ID NO: 12 is also encompassed. Yet a further alternative nucleic acid sequence encodes a soluble polypeptide having an amino acid sequence sufficiently duplicative of the soluble adenylyl cyclase encoded by SEQ ID NO: 1 or SEQ ID NO: 11 so that a polypeptide expressed from the nucleic acid molecule has the biological property of catalyzing the production of cyclic AMP, which polypeptide has a catalytic domain having a sequence that is not more than 16% similar to a catalytic domain of a mammalian transmembrane adenylyl cyclase as determined by CLUSTAL analysis.

In one embodiment the soluble adenylyl cyclase is mammalian soluble adenylyl cyclase. In a preferred embodiment the adenylyl cyclase is human soluble adenylyl cyclase; rat soluble adenylyl cyclase is also provided.

The invention further provides a vector comprising the nucleic acid molecule as defined above. The vector can be an expression vector, in which the nucleotide sequence encoding soluble adenylyl cyclase is operably associated with an expression control sequence, *e.g.*, for expression in human cells.

The invention further provides a host cell, preferably a mammalian cell, which comprises said expression vector.

The present invention also provides a method for producing recombinant soluble adenylyl cyclase comprising isolating soluble adenylyl cyclase expressed by a host cell containing an expression vector encoding a soluble adenylyl cyclase. In one embodiment, the soluble adenylyl cyclase is isolated using an anti-soluble adenylyl cyclase antibody.

The discovery of soluble adenylyl cyclase provides a mechanism for screening factors which modulate soluble adenylyl cyclase induced signaling. Thus the present invention provides a method of screening for a modulator of soluble adenylyl cyclase-induced signaling, which method comprises detecting inhibition of a signal of a soluble adenylyl cyclase-induced signal transduction pathway in a cell in the presence of a candidate compound wherein detection of inhibition of the signal indicates that the candidate compound is an inhibitor of soluble adenylyl cyclase-induced signaling. In one embodiment of the invention, the signal is cAMP generation. In this manner, cell proliferation, cell differentiation and apoptosis, control of which are diminished in pathological conditions such as cancer, can be modulated. Such modulation may have therapeutic and prophylactic benefit for those subjects suffering from such pathological conditions. Current treatment for these conditions, including chemotherapy and radiation therapy, are typically nonspecific and often have deleterious side effects. The identification of novel agents directed to a specific target to treat such conditions would be greatly advantageous.

The present invention also provides a method of modulating cAMP production by modulating the expression of soluble adenylyl cyclase. In this manner, for example, aberrant cell proliferation can be decreased or inhibited by regulating or modulating cAMP production.

With respect to decreasing cell proliferation, the present invention provides a method for decreasing or inhibiting soluble adenylyl cyclase expression such that cAMP production is decreased or inhibited and cell proliferation is decreased or inhibited.

Accordingly the present invention provides a method of regulating certain medical or pathological conditions in which cAMP production is implicated.

The present invention further provides a method of identifying factors which inhibit soluble adenylyl cyclase activity either by blocking expression of the soluble adenylyl cyclase gene or down regulating its ability to regulate cAMP metabolism. Such

factors can thus be used *in vivo* or *in vitro* to regulate soluble adenylyl cyclase activity.

The present invention also provides a method for modulating soluble adenylyl activity by increasing soluble adenylyl cyclase activity. Soluble adenylyl cyclase can be activated or its activity can be increased when stimulation of cAMP production is
5 desired. The present invention further provides a method of identifying factors that stimulate or enhance soluble adenylyl cyclase activity.

Applicants have specifically found that soluble adenylyl cyclase is potently stimulated by sodium bicarbonate. Controlling intracellular or extracellular bicarbonate concentrations provides an additional mechanism through which soluble adenylyl cyclase
10 activity, and ensuing cAMP production, can be regulated.

In this regard, applicants have found that regulating cAMP concentrations by modulating soluble adenylyl cyclase activity can provide a means of modulating sperm capacitation. The present invention provides a method of regulating sperm capacitation by regulating expression of soluble adenylyl cyclase. In one embodiment, the invention
15 provides a method for reducing or inhibiting male germ cell fertility by reducing or inhibiting soluble adenylyl cyclase activity and thereby inhibiting or decreasing capacitation of sperm. In one aspect the method comprises administering to a subject an agent that inhibits soluble adenylyl cyclase activity in an amount effective to decrease or inhibit cAMP production. In this manner, the fertilization of an ovum can be inhibited.

20 In another embodiment, the invention provides a method for increasing or enhancing the ability of sperm to fertilize an egg. With respect to this embodiment, the present invention provides a method of increasing sAC activity by, for example, treating sperm with a small molecule agonist, or using gene therapy to stimulate or enhance sperm capacitation. In this manner, the likelihood of fertilization of an ovum is increased or
25 enhanced. This is particularly useful in procedures such as *in vitro* fertilization.

Applicants have also found that regulating cAMP concentrations by regulating soluble adenylyl cyclase expression provides a means of regulating insulin secretion of pancreatic islet cells. cAMP is needed for the release of insulin (Liang Y et al., Annual Review of Nutrition, 1994, 14:59-81). Addition of bicarbonate activates
30 soluble adenylyl cyclase to increase cAMP production. With the discovery and isolation of soluble adenylyl cyclase, applicants provide a novel means of increasing or stimulating insulin release from the pancreas, when normal physiological mechanisms fail to do so, by

increasing soluble adenylyl cyclase activity.

Thus, the invention provides a method of increasing insulin secretion of pancreatic islet cells comprising increasing soluble adenylyl cyclase activity.

The present invention further provides a method of treating glaucoma by
5 reducing aqueous humor formation. With respect to this embodiment, aqueous humor formation, which is stimulated by cAMP signaling, can be reduced or inhibited by administering to a subject afflicted with glaucoma a modulator of sAC activity in an amount effective to reduce or inhibit bicarbonate dependent sAC activity to decrease the production of cAMP.

10 Applicants have also discovered that bicarbonate activates sAC in a direct, specific and pH independent manner. Accordingly, the present invention provides a method of quantifying bicarbonate in a body fluid using soluble adenylyl cyclase. The body fluid can be blood, urine, aqueous humor and the like.

In one aspect, the method of quantifying comprises contacting the body
15 fluid with sAC. Contact of bicarbonate with sAC activates sAC and generates cAMP. The amount of bicarbonate in the sample can be correlated to the amount of cAMP detected. Measurement or detection of cAMP can be effected through a number of means including fluorescence, colorimetry and the like. Due to its specificity for sAC, a direct correlation to the amount of bicarbonate in the sample body fluid can be made using this
20 method.

Applicants have further discovered that isolated soluble adenylyl cyclase is an oncogene. The isolated soluble adenylyl cyclase of the present invention is able to transform fibroblasts *in vitro* leading to loss of contact inhibition. Isolated soluble adenylyl cyclase and a truncated form of soluble adenylyl cyclase (sAC_T), transfected into
25 cells transformed NIH3T3 cells. Soluble adenylyl cyclase was further demonstrated to support anchorage independent growth in soft agar. The Ras related protein, Rap1 which is a specific competitive inhibitor of Ras, inhibited soluble adenylyl cyclase transformation of cells. Thus soluble adenylyl cyclase provides a target for Ras family (or other small GTPases) regulation of cAMP metabolism in mammals. This discovery is surprising
30 inasmuch that stimulation of transmembrane adenylyl cyclase activity blocks transformation of fibroblasts by oncogenic Ras (Chen, J., *et al.*, Science, 1994, 263:1278-81; Smit, M.J. *et al.*, Proc., Natl. Acad. Sci. USA, 1998, 95:15084-9).

Thus, the invention provides a method of inhibiting unwanted cell proliferation in an animal, a mammal, a human by administering an effective amount of a soluble adenylyl cyclase binding protein peptide fragment wherein the protein inhibits soluble adenylyl cyclase expression. In another aspect, the functional activity of soluble adenylyl cyclase can be inhibited by administering a specific soluble adenylyl cyclase antisense molecule to cells that express functional soluble adenylyl cyclase.

The observation that soluble adenylyl cyclase is expressed in tumor cells further provides a diagnostic marker to detect the presence of pathological conditions in an animal, *e.g.*, a mammal. In accordance with this aspect, the present invention provides a method of diagnosing the onset of, or the likelihood of onset of, or for monitoring the course and severity of a pathological condition derived from soluble-adenylyl cyclase activation, comprising detecting an increase in soluble adenylyl cyclase levels in a biological sample obtained from a subject suspected of suffering from such conditions.

The invention further provides compositions and kits for the diagnosis of conditions arising from soluble-adenylyl cyclase activation.

These and other aspects of the invention are more fully set forth in the Drawings, Detailed Description, and Examples.

DESCRIPTION OF THE FIGURES

Figures 1a and 1b show the predicted amino acid sequence of rat sAC (SEQ ID NO: 1). Amino acids in bold indicate presumptive catalytic domains, C1 and C2. Double-underlined amino acids correspond to sequences of tryptic peptides derived from the purified 48-kDa protein. Dotted underlined amino acids conform to a consensus P loop sequence, and underlined sequences are predicted to form a leucine zipper. Valine 469 is underlined and is the last amino acid in the catalytically active heterologously expressed truncated sAC (sAC_T).

Figure 2 shows that anti-sAC antiserum specifically recognizes heterologously expressed short and long forms of sAC. Whole cell lysates of HiFive cells infected with baculovirus vector (–), or with recombinant baculovirus expressing Truncated (T) or Full-length (F) sAC were separated on a 7.5% SDS/PAGE and Western blotted with the indicated crude antiserum. “αC1-C2” refers to affinity purified anti-catalytic (N-terminal ~50 kD) antiserum. Detection was by enhanced chemiluminescence

(Pierce); exposure times were all less than one minute.

Figure 3 shows multiple sAC isoforms in staged germ cell preparation. Whole cell lysates of the indicated enriched germ cell populations were separated on a 7.5% SDS/PAGE and Western blotted with the affinity purified antiserum: C = control samples representing a mixture of extracts from Hi5 cells infected with truncated (50 kD) and full-length expressing baculovirus, partially degraded; RS = round spermatids; PS = pachytene spermatocytes; and S = spermatozoa. Arrows point out sAC_n, 120 kD, 48 kD, and 45 kD isoforms. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce); exposure times were less than one minute.

Figures 4a and b show that sAC is detected in a number of bicarbonate sensing tissues. Figure 4a- Anti-sAC Western blot of testis (30 µg), sperm (5 µg), kidney (50 µg), and choroid plexus (50 µg). Figure 4b- Adenylyl cyclase activity in immunoprecipitates from testis cytosol using either pre-immune serum or α-sAC antisera.

Figures 5a and b show that sAC is stimulated by bicarbonate. Figure 5a- Cellular cAMP accumulation was measured in stable cell lines expressing expression vector alone (diamonds) or sAC_n (squares) at the indicated concentrations of NaHCO₃ following growth for 24 hours under bicarbonate-free conditions. Data are expressed as cAMP formed as a percentage of total adenine nucleotides, and values represent averages of quadruplicate determinations with standard deviations indicated. Figure 5b- *In vitro* cyclase activity in extracts from stable cell lines expressing empty expression vector, sAC_n, or sAC_t in the presence (dark bars) or absence (light bars) of 50 mM NaHCO₃. Data are expressed as pmol of cAMP formed per minute per mg total protein, and values represent averages of triplicate determinations with standard deviations indicated.

Figures 6a, b, and c show that bicarbonate activation of sAC is direct, specific, and pH independent. Figure 6a- Purified sAC_t was assayed in the presence of a range of concentrations of NaHCO₃ (0-80 mM) with 10 mM ATP and 40 mM MgCl₂. Data are expressed as nmol cAMP formed per minute per mg protein, and values are averages of triplicate determinations. Figure 6b- Purified sAC_t was assayed at the indicated final pHs (buffered by Tris-HCl) in the presence (circles) or absence (squares) of 40 mM NaHCO₃. Best fit lines were generated using linear regression analysis. Figure 6c- Purified recombinant soluble tmACV was assayed in the presence of MgCl₂ alone (basal), or with 50 mM NaHCO₃, or 100 µM Forskolin. Data are presented as pmol cAMP formed

per minute per mg protein, and values represent triplicate determinations with standard deviations indicated.

Figures 7a and b show the relationship between sACs and that bicarbonate activates cyanobacterial adenylyl cyclase. Figure 7a- Phylogenetic relationship between catalytic domains from a variety of ACs aligned using CLUSTALW (DNA*) represented as an unrooted dendrogram constructed using PROTPARS (PHYLIP 3.5). Numbers represent bootstrap confidence values. Accession numbers for the aligned amino acid sequences are sAC [rat sAC: AAD04035] tmAC1 [bovine Type 1: AAA799571, tmAC2 [rat Type 11: AAA40682], tmAC5 [rat Type V: Q04400], tmAC9 [mouse Type IX: CAA03415], D.d. AcrA [*Dictyostelium discoideum* AcrA: AAD50121], Asp. (*Anabaena spirulina*) cyaA [BAA13997], A.sp. cyaB1 [BAA139981, A.sp. cyaB2 [BAA13999], A.sp. cyaC [BAA14000], A.sp. cyaD [BAA140011, S.pl. (*Spirulina platensis*) CyaA [BAA22996], S.pl. CyaC [BAA22997], Syn. (*Synechocystis sp.*) CyaA1 [BAA16969], and Syn. CyaA2 [BAA17880]. Figure 7b-Expressed and purified *Spirulina platensis* CyaC was assayed in the presence of a range of concentrations of NaHCO₃ (0-60 mM) with 100 μM ATP and 5 mM MnCl₂. Data are expressed as pmol cAMP formed per minute per mg protein, and values are averages of triplicate determinations.

Figures 8a to 8f are photographs of Giemsa-stained foci formed in NIH3T3 cells transfected with vector alone (Fig. 8a), v-Ras (Fig. 8b), truncated soluble adenylyl cyclase (sAC_t)(Fig. 8c), full length soluble adenylyl cyclase (sAC_n)(Fig. 8d), transmembrane adenylyl cyclase Type II (tmAC2)(Fig. 8e), or constitutively active Gsα* protein (Fig. 8f).

Figure 9 shows sAC expression in human colon carcinoma compared to normal colon. Western blot analysis with indicated sAC-specific antisera against normal colon mucosa (M), colon carcinoma (T), or in one case, a biopsied lymph node (LN) from three, independent cancer patients. sAC protein is specifically unregulated in patients #1 and #2.

DETAILED DESCRIPTION

In accordance with the present invention, a distinct class of soluble adenylyl cyclases is presented. Applicants have surprisingly discovered that soluble adenylyl cyclase (sAC) is expressed not only in testis, but throughout animal tissue types including

brain, kidney, skeletal muscle, liver, lung, spleen and heart. sAC activity has been detected in sea urchin sperm.

The present invention is based, in part, on the purification, cloning and characterization of soluble animal adenylyl cyclase (sAC). sAC was purified from cytosolic extracts of rat testes. This catalytically active purified form of sAC was identified as a 48-kD protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purified 48 kD species corresponds to the N-terminal of the predicted protein. The sAC activity was only detected in vitro in the presence of Mn^{2+} -ATP and was unresponsive to either forskolin or GTP γ S. However, sAC activity was stimulated by bicarbonate in the presence of Mg^{2+} -ATP.

The full length cDNA predicts a protein of about 187 kD. Full-length sAC is proteolytically processed into multiple developmentally regulated isoforms postulated to serve distinct cellular functions. At least five isoforms have been identified having apparent molecular weights of 190 kD, 150 kD, 120 kD, 48 kD and 45 kD as determined by SDS-PAGE, with detection by sAC specific antisera described herein. The expression of sAC appears to be regulated during cell development (for example, in sperm) and varies in expression between individual cell lines.

It is contemplated as part of the present invention that sAC can be isolated from body tissue or can be produced by recombinant DNA methods or synthetic peptide chemical methods that are well known to those of ordinary skill in the art.

sAC Nucleic Acid and Protein

The sAC gene was identified in rat, mouse, pig, and human and encodes a cytosolic form of adenylyl cyclase that is distinct from the previously characterized mammalian tmACs. Not only is sAC not a transmembrane protein, but its catalytic domains are more closely related to the catalytic portions of bacterial ACs than they are to the catalytic domains of any other eukaryotic cyclase. In contrast, the mammalian tmACs, which are distantly related to these bacterial ACs and sAC, more closely resemble other invertebrate (*Drosophila*) and lower eukaryotic (*Dictyostelium*) ACs. The amino acid sequence of the isolated rat sAc molecule is shown in Fig. 1 and in SEQ ID NO: 1. The amino-terminal portion of approximately 50 kD, encompassing both catalytic domains C1 and C2 is sufficient for sAC enzymatic activity. Using the CLUSTAL method of sequence

comparison provided with the MEGALIGN program manufactured by DNA Star, and using standard default parameters, sAC C1 and C2 are 24.3% similar to each other. Both C1 and C2 are only between 10-16% similar to the catalytic domains of the various mammalian transmembrane adenylyl cyclases. C1 is 14.1% similar to yeast
5 (*Saccharomyces cerevisiae*) adenylyl cyclase. C2 is 14.9% similar to yeast (*Saccharomyces cerevisiae*) adenylyl cyclase.

In contrast, C1 is 35.9% similar to (Cyanobacterial) *Anabaena spirulensis* CyaB1 and 33.2% similar to *Anabaena spirulensis* CyaA, while C2 is 25.9% similar to *Anabaena spirulensis* CyaA and 27.7% similar to *Anabaena spirulensis* CyaB1. A sAC-
10 like cyclase has also been identified in cyanobacteria. Cyanobacteria are gram negative bacteria that, in the presence of sunlight, water and carbon dioxide, perform oxygen-evolving photosynthesis similar to plants. They respond to changes in environmental availability of these raw materials, such as shifts between light and dark and between low pH and high pH, via changes in their intracellular cAMP concentration (Ohmori, Plant
15 Cell Physiol., 1989, 30:911-914; Ohmori *et al.*, Arch. Microbiol., 1988, 150:203-204), and membrane permeable cAMP analogs stimulate metabolism, i.e. the activity of respiration, in the cyanobacteria *Spirulina platensis* (Ohmori *et al.*, Plant Cell Physiol., 1992, 33:21-25). Cyanobacterial adenylyl cyclases may provide the link between light sensation and cAMP generation. Genetic disruption of a particular adenylyl cyclase isoform causes loss
20 of the light-dark response in the cyanobacterium *Anabaena* (Katayama and Ohmori, J. Bacteriol., 1997, 179:3588-93) and the cyaC adenylyl cyclase from *Spirulina platensis* is thought to be directly stimulated by light via its histidine kinase phosphotransfer regulatory domain (Kasahara *et al.*, Plant Cell Physiol., 1997, 38:828-36; Kasahara and Ohmori, 1999, J. Biol. Chem. 274:15167-15172). Applicants have found that cyaC is
25 directly stimulated by bicarbonate.

The anti-catalytic domain (C1-C2) antisera on tested sea urchin sperm membranes and on a partially purified fraction containing sea urchin sperm adenylyl cyclase activity. The results of a Western blot show, particularly in the partially purified sperm cyclase containing fraction, a band at 190 kD, which is the size predicted for sea
30 urchin sperm cyclase (Bookbinder LH *et al.*, J. Biol. Chem., 1990, 111:1859-1866). In addition, an immunoreactive band at approximately 120 kD was detected, suggesting that proteolytic processing leading to distinct sAC isoforms is also conserved between species.

A soluble adenylyl cyclase is a protein having an amino acid sequence substantially similar to that of the isolated sAC described herein having sAC activity. The term substantially similar when used in reference to sAC amino acid sequences means an amino acid sequence having sAC activity and having a molecular weight of approximately
5 187 kD.

The amino acid sequence of the sAC of the present invention may vary depending on which isoform cell or tissue the sAC is derived.

It is also contemplated that the molecule sAC can be a fragment of the full length sAC molecule, having sAC activity. In one embodiment a sAC can have a
10 molecular weight of approximately 48-53 kD and an amino acid sequence substantially similar to the amino terminal portion of the rat sAC sequence SEQ ID NO: 1. The soluble adenylyl cyclase can be a chimeric variant or modified derivatives, including a chemically modified derivative including modification by PEGylation. The human sAC locus has been sequenced as part of the Genome Project. It is encoded by more than 30 exons that
15 are spread across two overlapping PAC (P1-derived artificial chromosome) clones mapping to 1q24 human PAC clones. GenBank reference Accession Numbers HS295C6 Human DNA sequence from PAC 295C6 on chromosome 1Q24, HS313L4 Human DNA sequence from PAC 313L4 on chromosome 1q24. The human sAC amino acid sequence is set out as the sequence of SEQ ID NO: 11; the DNA sequence is set out as the sequence
20 of SEQ ID NO: 12.

A nucleic acid or polypeptide sequence that is "derived from" a designated sequence refers to a sequence that corresponds to a region of the designated sequence. For nucleic acid sequences, this encompasses sequences that are homologous or complementary to the sequence, as well as "sequence-conservative variants" and
25 "function-conservative variants."

"Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position.

"Function-conservative variants" are those in which a given amino acid
30 residue in a protein or enzyme has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, polarity, hydrogen bonding

potential, acidic, basic, hydrophobic, aromatic, and the like). Amino acids with similar properties are well known in the art. For example, arginine, histidine and lysine are hydrophilic-basic amino acids and may be interchangeable. Similarly, isoleucine, a hydrophobic amino acid, may be replaced with leucine, methionine or valine. Such changes are expected to have little or no effect on the apparent molecular weight or isoelectric point of the protein or polypeptide. Amino acids other than those indicated as conserved may differ in a protein or enzyme so that the percent protein or amino acid sequence similarity between any two proteins of similar function may vary and may be, for example, from 70% to 99% as determined according to an alignment scheme such as by the Clustal Method, wherein similarity is based on the MEGALIGN algorithm. A "function-conservative variant" also includes a polypeptide or enzyme which has at least 60 % amino acid identity as determined by BLAST or FASTA algorithms, preferably at least 75%, most preferably at least 85%, and even more preferably at least 90%, and which has the same or substantially similar properties or functions as the native or parent protein or enzyme to which it is compared. Finally, for purposes of the invention, a functional-conservative variant includes a truncated form of the protein that performs its function, or splice variants, or proteolytic fragments of the proteins such as sAC isoforms characterized by molecular weights 190 kD, 150 kD, 120 kD, 48 kD and 45 kD. Functional-conservative variants also include any polypeptides that have the ability to elicit antibodies specific to a designated polypeptide.

Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 80% of the amino acids are identical, or greater than about 90% are similar (functionally identical).

Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin) pileup program, or any of the programs described above (BLAST, FASTA),

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook *et al.*, *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency

hybridization conditions, corresponding to a T_m (melting temperature) of 55°C, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m , *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m , *e.g.*, 50% formamide, 5x or 6x SCC. SCC is a 0.15M NaCl, 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see Sambrook et al., supra*, 9.50-9.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see Sambrook et al., supra*, 11.7-11.8). A minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; preferably at least about 15 nucleotides; and more preferably the length is at least about 20 nucleotides.

In a specific embodiment, the term "standard hybridization conditions" refers to a T_m of 55°C, and utilizes conditions as set forth above. In a preferred embodiment, the T_m is 60°C; in a more preferred embodiment, the T_m is 65°C. In a specific embodiment, "high stringency" refers to hybridization and/or washing conditions at 68°C in 0.2XSSC, at 42°C in 50% formamide, 4XSSC, or under conditions that afford levels of hybridization equivalent to those observed under either of these two conditions.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 10, preferably at least 15, and more preferably at least 20 nucleotides, preferably no more than 100 nucleotides, that is hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labeled, *e.g.*, with ^{32}P -nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one

embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of sAC, or to detect the presence of nucleic acids encoding sAC. In a further embodiment, an
5 oligonucleotide of the invention can form a triple helix with a sAC DNA molecule. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds, etc.

The present invention provides antisense nucleic acids (including
10 ribozymes), which may be used to inhibit expression of sAC of the invention, particularly to suppress sAC regulation of cAMP. An "antisense nucleic acid" is a single stranded nucleic acid molecule which, on hybridizing under cytoplasmic conditions with complementary bases in an RNA or DNA molecule, inhibits the latter's role. If the RNA is a messenger RNA transcript, the antisense nucleic acid is a countertranscript or mRNA-
15 interfering complementary nucleic acid. As presently used, "antisense" broadly includes RNA-RNA interactions, RNA-DNA interactions, ribozymes and RNase-H mediated arrest. Antisense nucleic acid molecules can be encoded by a recombinant gene for expression in a cell (*e.g.*, U.S. Patent No. 5,814,500; U.S. Patent No. 5,811,234), or alternatively they can be prepared synthetically (*e.g.*, U.S. Patent No. 5,780,607).

20 Specific non-limiting examples of synthetic oligonucleotides envisioned for this invention include oligonucleotides that contain phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl, or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are those with CH₂-NH-O-CH₂, CH₂-N(CH₃)-O-CH₂, CH₂-O-N(CH₃)-CH₂, CH₂-N(CH₃)-N(CH₃)-CH₂ and O-N(CH₃)-CH₂-CH₂ backbones (where phosphodiester is O-PO₂-O-CH₂). US Patent No.
25 5,677,437 describes heteroaromatic oligonucleoside linkages. Nitrogen linkers or groups containing nitrogen can also be used to prepare oligonucleotide mimics (U.S. Patents No. 5,792,844 and No. 5,783,682). US Patent No. 5,637,684 describes phosphoramidate and phosphorothioamidate oligomeric compounds. Also envisioned are oligonucleotides
30 having morpholino backbone structures (U.S. Pat. No. 5,034,506). In other embodiments, such as the peptide-nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide may be replaced with a polyamide backbone, the bases being bound

directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen *et al.*, Science 254:1497, 1991). Other synthetic oligonucleotides may contain substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH₃, F, OCN, O(CH₂)_nNH₂ or O(CH₂)_nCH₃ where n is from 1 to about 10; C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF₃; OCF₃; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂CH₃; ONO₂; NO₂; N₃; NH₂; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; a fluorescein moiety; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Oligonucleotides may also have sugar mimetics such as cyclobutyls or other carbocyclics in place of the pentofuranosyl group. Nucleotide units having nucleosides other than adenosine, cytidine, guanosine, thymidine and uridine, such as inosine, may be used in an oligonucleotide molecule.

15

General Definitions

As used herein, the term "isolated" means that the soluble adenylyl cyclase is removed from the environment in which it is normally found. Thus, an isolated biological material can be free of cellular components, *i.e.*, components of the cells in which the material is found or produced. In the case of nucleic acid molecules, an isolated nucleic acid includes a PCR product, an isolated mRNA, a cDNA, or a restriction fragment. In another embodiment, an isolated nucleic acid is preferably excised from the chromosome in which it may be found, and more preferably is no longer joined to non-regulatory, non-coding regions, or to other genes, located upstream or downstream of the gene contained by the isolated nucleic acid molecule when found in the chromosome. In yet another embodiment, the isolated nucleic acid lacks one or more introns. Isolated nucleic acid molecules include sequences inserted into plasmids, cosmids, artificial chromosomes, and the like. Thus, in a specific embodiment, a recombinant nucleic acid is an isolated nucleic acid. An isolated protein may be associated with other proteins or nucleic acids, or both, with which it associates in the cell, or with cellular membranes if it

is a membrane-associated protein. An isolated organelle, cell, or tissue is removed from the anatomical site in which it is found in an organism. An isolated material may be, but need not be, purified.

The term "purified" as used herein refers to material that has been isolated
5 under conditions that reduce or eliminate the presence of unrelated materials, *i.e.*,
contaminants, including native materials from which the material is obtained. For
example, a purified protein is preferably substantially free of other proteins or nucleic
acids with which it is associated in a cell; a purified nucleic acid molecule is preferably
substantially free of proteins or other unrelated nucleic acid molecules with which it can be
10 found within a cell. As used herein, the term "substantially free" is used operationally, in
the context of analytical testing of the material. Preferably, purified material substantially
free of contaminants is at least 50% pure; more preferably, at least 90% pure, and more
preferably still at least 99% pure. Purity can be evaluated by chromatography, gel
electrophoresis, immunoassay, composition analysis, biological assay, and other methods
15 known in the art.

Protein purification methods are well known in the art and a specific
example of a method for purifying sAC is provided in the examples below. For example,
nucleic acids can be purified by precipitation, chromatography (including preparative solid
phase chromatography, oligonucleotide hybridization, and triple helix chromatography),
20 ultracentrifugation, and other means. Polypeptides and proteins can be purified by various
methods including, without limitation, preparative disc-gel electrophoresis, isoelectric
focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition
chromatography, precipitation and salting-out chromatography, extraction, and
countercurrent distribution. For some purposes, it is preferable to produce the polypeptide
25 in a recombinant system in which the protein contains an additional sequence tag that
facilitates purification, such as, but not limited to, a polyhistidine sequence, or a sequence
that specifically binds to an antibody, such as FLAG and GST. The polypeptide can then
be purified from a crude lysate of the host cell by chromatography on an appropriate
solid-phase matrix. Alternatively, antibodies produced against the protein or against
30 peptides derived therefrom can be used as purification reagents. Cells can be purified by
various techniques, including centrifugation, matrix separation (*e.g.*, nylon wool
separation), panning and other immunoselection techniques, depletion (*e.g.*, complement

depletion of contaminating cells), and cell sorting (*e.g.*, fluorescence activated cell sorting (FACS)). Other purification methods are possible. A purified material may contain less than about 50%, preferably less than about 75%, and most preferably less than about 90%, of the cellular components with which it was originally associated. The "substantially
5 pure" indicates the highest degree of purity which can be achieved using conventional purification techniques known in the art.

In a specific embodiment, the term "about" or "approximately" means within 20%, preferably within 10%, and more preferably within 5% of a given value or range. Alternatively, particularly in biology, the term "about" can mean within an order of
10 magnitude of a given value, and preferably within one-half an order of magnitude of the value.

The term "inhibitor" is used herein to refer to a compound that can block signaling in the signal transduction pathway described herein. Such an inhibitor may directly affect sAC function, substrate recognition, or activation. Preferably, an inhibitor
15 discovered in accordance with the invention is specific for signals of sAC-induced signaling. Such an inhibitor may also be termed an antagonist.

The term "agonist" is used herein to refer to a compound that can induce signaling in the signal transduction pathway described herein. Preferably an agonist discovered in accordance with the invention is specific for signals of sAC-induced
20 signaling.

"Screening" refers to a process of testing one or a plurality of compounds (including a library of compounds) for some activity. A "screen" is a test system for screening. Screens can be primary, *i.e.*, an initial selection process, or secondary, *e.g.*, to confirm that a compound selected in a primary screen (such as a binding assay) functions
25 as desired (such as in a signal transduction assay). Screening permits the more rapid elimination of irrelevant or non-functional compounds, and thus selection of more relevant compounds for further testing and development. "High throughput screening" involves the automation and robotization of screening systems to rapidly screen a large number of compounds for a desired activity.

30

Molecular Biology - Definitions

In accordance with the present invention there may be employed

conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. (1985)); *Transcription And Translation* (B.D. Hames & S.J. Higgins, eds. (1984)); *Animal Cell Culture* (R.I. Freshney, ed. (1986)); *Immobilized Cells And Enzymes* (IRL Press, (1986)); B.ÉPerbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

The term "host cell" means any cell of any organism that is selected, modified, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression by the cell of a gene, a DNA or RNA sequence, a protein or an enzyme. Host cells can further be used for screening or other assays, as described *infra*.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, *i.e.*, the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

An "opening reading frame" (ORF) as used herein is a region of a polynucleotide sequence having a start and codon and which may encode a polypeptide. This region may represent a portion of a coding sequence or may comprise a total coding sequence for the polypeptide.

A "complement" of a nucleic acid sequence as used herein refers to the "antisense" sequence that participates in Watson-Crick base-pairing with the original

sequence.

The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids, which comprise all or part of one or more proteins or enzymes. A gene as used herein may or may not include
5 non-transcribed regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. Furthermore, a transcribed portion of the gene may include 5'- and 3'-untranslated sequences and introns in addition to the coding sequence.

A "promoter sequence" is a DNA regulatory region capable of binding
10 RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be
15 found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" or "operatively (or operably) associated with" of transcriptional and translational control sequences in a cell when RNA
20 polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if it contains introns) and translated into the protein encoded by the coding sequence.

The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a
25 corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, *e.g.* the resulting protein, may also be said to be "expressed" by the cell. An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means something that is inside a cell. The term "extracellular" means something that is
30 outside a cell. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

The term "transfection" means the introduction of a foreign nucleic acid

into a cell. The term "transformation" means the introduction of a "foreign" (*i.e.* extrinsic or extracellular) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. The introduced gene or sequence
5 may also be called a "cloned" or "foreign" gene or sequence, may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone." The DNA or
10 RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (*e.g.* a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (*e.g.* transcription and
15 translation) of the introduced sequence. Vectors include plasmids, phages, viruses, etc.; they are discussed in greater detail below.

The term "expression system" means a host cell and compatible vector under suitable conditions, *e.g.* for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. Expression systems include
20 mammalian host cells and vectors. Suitable cells include C12 cells, CHO cells, HeLa cells, 293 and 293T (human kidney cells), mouse primary myoblasts, and NIH 3T3 cells.

Vectors

A wide variety of host/expression vector combinations may be employed in expressing DNA sequences encoding sAC other proteins involved in cAMP signaling, or
25 inhibitors of sAC such as antisense nucleic acids or anti-sAC intracellular antibodies. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, *e.g.*, *E. coli* plasmids col E1, pCR1, pBR322, pMal-C2, pET, pGEX (Smith *et al.*, Gene 67:31-40, 1988), pMB9 and their derivatives,
30 plasmids such as RP4; phage DNAs, *e.g.*, the numerous derivatives of phage λ , *e.g.*, NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA;

yeast plasmids such as the 2 μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

5 Expression of the protein or polypeptide may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist and
10 Chambon, *Nature* 290:304-310, 1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, *Cell* 22:787-797, 1980), the herpes thymidine kinase promoter (Wagner, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445, 1981), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, *Nature* 296:39-42, 1982); prokaryotic expression vectors such as the β -lactamase promoter (Villa-
15 Komaroff, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 75:3727-3731, 1978), or the *tac* promoter (DeBoer, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 80:21-25, 1983); see also "Useful proteins from recombinant bacteria" in *Scientific American*, 242:74-94, 1980; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and
20 control regions that exhibit hematopoietic tissue specificity, in particular: immunoglobulin gene control region, which is active in lymphoid cells (Grosschedl *et al.*, *Cell*, 38:647, 1984; Adames *et al.*, *Nature*, 318:533, 1985; Alexander *et al.*, *Mol. Cell Biol.*, 7:1436, 1987); beta-globin gene control region which is active in myeloid cells (Mogam, *et al.*, *Nature* 315:338-340, 1985; Kollias, *et al.*, *Cell* 46:89-94, 1986), hematopoietic stem cell
25 differentiation factor promoters; erythropoietin receptor promoter (Maouche, *et al.*, *Blood*, 15:2557, 1991), etc; and control regions that exhibit mucosal epithelial cell specificity.

A vector can be introduced *in vivo* in a non-viral vector, *e.g.*, by lipofection, with other transfection facilitating agents (peptides, polymers, etc.), or as naked DNA. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection, with
30 targeting in some instances (Felgner, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84:7413-7417, 1987; Felgner and Ringold, *Science* 337:387-388, 1989; see Mackey, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 85:8027-8031, 1988; Ulmer *et al.*, *Science* 259:1745-1748, 1993).

Useful lipid compounds and compositions for transfer of nucleic acids are described in International Patent Publications WO95/18863 and WO96/17823, and in U.S. Patent No. 5,459,127. Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, International Patent Publication WO95/21931),
5 peptides derived from DNA binding proteins (*e.g.*, International Patent Publication WO96/25508), or a cationic polymer (*e.g.*, International Patent Publication WO95/21931). Recently, a relatively low voltage, high efficiency *in vivo* DNA transfer technique, termed electrotransfer, has been described (Mir *et al.*, C.P. Acad. Sci., 321:893, 1998; WO 99/01157; WO 99/01158; WO 99/01175). DNA vectors for gene therapy can be
10 introduced into the desired host cells by methods known in the art, *e.g.*, electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun (ballistic transfection), or use of a DNA vector transporter (see, *e.g.*, Wu *et al.*, J. Biol. Chem. 267:963-967, 1992; Wu and Wu, J. Biol. Chem. 263:14621-14624, 1988; Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990; Williams *et al.*,
15 Proc. Natl. Acad. Sci. USA 88:2726-2730, 1991). Receptor-mediated DNA delivery approaches can also be used (Curiel *et al.*, Hum. Gene Ther. 3:147-154, 1992; Wu and Wu, J. Biol. Chem. 262:4429-4432, 1987). US Patent Nos. 5,580,859 and 5,589,466 disclose delivery of exogenous DNA sequences, free of transfection facilitating agents, in a mammal.

20 Also useful are viral vectors, such as lentiviruses, retroviruses, herpes viruses, adenoviruses, adeno-associated viruses, vaccinia virus, baculovirus, and other recombinant viruses with desirable cellular tropism. Thus, a gene encoding a functional protein or polypeptide (as set forth above) can be introduced *in vivo*, *ex vivo*, or *in vitro* using a viral vector or through direct introduction of DNA. Expression in targeted tissues
25 can be effected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both. Targeted gene delivery is described in International Patent Publication WO 95/28494, published October 1995.

Viral vectors commonly used for *in vivo* or *ex vivo* targeting and therapy
30 procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art (*see, e.g.*, Miller and Rosman, BioTechniques, 7:980-990, 1992). Preferably, the viral vectors are replication defective, that is, they are

unable to replicate autonomously in the target cell. In general, the genome of the replication defective viral vectors which are used within the scope of the present invention lack at least one region which is necessary for the replication of the virus in the infected cell. These regions can either be eliminated (in whole or in part), be rendered non-
5 functional by any technique known to a person skilled in the art. These techniques include the total removal, substitution (by other sequences, in particular by the inserted nucleic acid), partial deletion or addition of one or more bases to an essential (for replication) region. Such techniques may be performed *in vitro* (on the isolated DNA) or *in situ*, using the techniques of genetic manipulation or by treatment with mutagenic agents. Preferably,
10 the replication defective virus retains the sequences of its genome which are necessary for encapsidating the viral particles.

DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely
15 or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a specific tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kaplitt *et al.*, Molec. Cell.
20 Neurosci. 2:320-330, 1991), defective herpes virus vector lacking a glycoprotein L gene (Patent Publication RD 371005 A), or other defective herpes virus vectors (International Patent Publication No. WO 94/21807, published September 29, 1994; International Patent Publication No. WO 92/05263, published April 2, 1994); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.* (J. Clin. Invest. 90:626-630,
25 1992; see also La Salle *et al.*, Science 259:988-990, 1993); and a defective adeno-associated virus vector (Samulski *et al.*, J. Virol. 61:3096-3101, 1987; Samulski *et al.*, J. Virol. 63:3822-3828, 1989; Lebkowski *et al.*, Mol. Cell. Biol. 8:3988-3996, 1988).

Various companies produce viral vectors commercially, including but by no means limited to Avigen, Inc. (Alameda, CA; AAV vectors), Cell Genesys (Foster City,
30 CA; retroviral, adenoviral, AAV vectors, and lentiviral vectors), Clontech (retroviral and baculoviral vectors), Genovo, Inc. (Sharon Hill, PA; adenoviral and AAV vectors), Genvec (adenoviral vectors), IntroGene (Leiden, Netherlands; adenoviral vectors),

Molecular Medicine (retroviral, adenoviral, AAV, and herpes viral vectors), Norgen (adenoviral vectors), Oxford BioMedica (Oxford, United Kingdom; lentiviral vectors), and Transgene (Strasbourg, France; adenoviral, vaccinia, retroviral, and lentiviral vectors).

Preferably, for *in vivo* administration, an appropriate immunosuppressive treatment is employed in conjunction with the viral vector, *e.g.*, adenovirus vector, to avoid immuno-deactivation of the viral vector and transfected cells. For example, immunosuppressive cytokines, such as interleukin-12 (IL-12), interferon- γ (IFN- γ), or anti-CD4 antibody, can be administered to block humoral or cellular immune responses to the viral vectors (*see, e.g.*, Wilson, Nature Medicine, 1995). In that regard, it is advantageous to employ a viral vector that is engineered to express a minimal number of antigens.

Antibodies to sAC

According to the invention, sAC polypeptides produced recombinantly or by chemical synthesis, and fragments or other derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize the sAC polypeptide. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. Such antibodies are specific for sAC or specific portions of sAC.

Various procedures known in the art may be used for the production of polyclonal antibodies to sAC polypeptide or derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with the sAC polypeptide, or a derivative (*e.g.*, fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, the sAC polypeptide or fragment thereof can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward the sAC polypeptide, or fragment, analog, or derivative thereof, any technique that provides for the

production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein (Nature 256:495-497, 1975), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, Immunology Today 4:72, 1983; Cote *et al.*, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030, 1983), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985). Production of human antibodies by CDR grafting is described in U.S. Patent Nos. 5,585,089, 5,693,761, and 5,693,762 to Queen *et al.*, and also in U.S. Patent No. 5,225,539 to Winter and International Patent Application PCT/WO91/09967 by Adau *et al.* In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals (International Patent Publication No. WO 89/12690, published 28 December 1989). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, J. Bacteriol. 159:870, 1984); Neuberger *et al.*, Nature 312:604-608, 1984; Takeda *et al.*, Nature 314:452-454, 1985) by splicing the genes from a mouse antibody molecule specific for a sAC polypeptide together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders, since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778) can be adapted to produce sAC polypeptide-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse *et al.*, Science 246:1275-1281, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for an sAC polypeptide, or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of

the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of a sAC polypeptide, one may assay generated hybridomas for a product which binds to a sAC polypeptide fragment containing such epitope. For selection of an antibody specific to a sAC polypeptide from a particular species of animal, one can select on the basis of positive binding with sAC polypeptide expressed by or isolated from cells of that species of animal.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the sAC polypeptide, *e.g.*, for Western blotting, imaging sAC polypeptide *in situ*, measuring levels thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned above or known in the art. Such antibodies can be used to identify proteins that interact with sAC.

In a specific embodiment, antibodies that agonize or antagonize the activity of sAC polypeptide can be generated. They can also be used to regulate or inhibit sAC activity intracellularly, *i.e.*, the invention contemplates an intracellular antibody (intrabody), *e.g.*, single chain Fv antibodies (*see generally*, Chen, Mol. Med. Today, 3:160-167, 1997; Spitz *et al.*, Anticancer Res., 16:3415-3422, 1996; Indolfi *et al.*, Nat. Med., 2:634-635, 1996; Kijima *et al.*, Pharmacol. Ther., 68:247-267, 1995).

The antibodies that specifically bind to the sAC or peptides or fragment,

thereof can be used in diagnostic methods and kits that are well known to those of skill in the art to detect or quantify sAC in bodily tissue. Results from these test can be used to diagnose or detect the occurrence of a pathological condition mediated by sAC expression, for example tumor formation.

5

sAC: Oncogene

sAC is a new oncogene that functions in the oncogenic signaling pathway of mammalian Ras proteins. sAC interacts with the mammalian ortholog of yeast CAP, which is required for Ras-responsiveness of yeast cyclase. When mammalian CAP is co-expressed with sAC, sAC activity increases approximately four-fold. In addition, two out of three human colon carcinomas, which often harbor oncogenic Ras mutations, when tested had elevated levels of sAC isoforms. sAC expression was upregulated in pre-cancerous tissue in Min mice, a model system for generic predisposition of colon cancer.

10

The identification of sAC as a unique form of mammalian adenylyl cyclase further provides a new potential target for Ras regulation of cAMP signaling in mammals.

15

A truncated form of sAC (sAC_t) was highly active and was able to transform NIH 3T3 cells by focus-forming assay. It produced as many foci as the oncogenic form of Ras. Oncogenic transformation of NIH 3T3 cells is often a consequence of uncontrolled cell growth (*i.e.*, by loss of contact inhibition) and/or decreased cell death (*i.e.*, by oncogenes such as p53, Rb, APC and Ras (Joneson, T. and D. Bar-Sagi (199), Mol. Cell Biol. 19: 5892-901; Leblanc, V et al., (1999) Oncogene 18: 4884-9)). Full-length sAC (sAC_{fl}) was less oncogenic, inducing more foci than vector alone, but less than sAC_t. We also found that sAC_t and sAC_{fl} support anchorage independent growth in soft agar.

20

Rap1 protein, which selectively blocks transformation by oncogenic Ras proteins, blocked transformation by sAC_t suggesting that sAC and Ras may share their biochemical mechanism of transformation.

25

sAC Modulators

The present invention further provides various screening assays for identifying sAC modulators, *i.e.*, inhibitors or agonists, and particularly sAC induced activation of cAMP, useful as targets for diagnosis and/or treatment of conditions arising from inappropriate activation or a deficiency of sAC. The screening assays of the

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invention are particularly advantageous by permitting rapid evaluation of cellular response. Biological assays, which depend on cell growth, survival, or some other response require substantial amounts of time and resources to evaluate. By detecting individual signals in the sAC-induced signal transduction pathway, the present invention short-circuits tedious and time consuming biological assays. Furthermore, signal transduction assays can often be performed with very small amounts of material.

The present invention contemplates screens for small molecule compounds, including ligand analogs and mimics, as well as screens for natural compounds that bind to and agonize or antagonize sAC signal transduction *in vivo*. Such agonists or antagonists may, for example, interfere in the signaling cascade induced by sAC generated cAMP. For example, natural products libraries can be screened using assays of the invention for such molecules. As used herein, the term "compound" refers to any molecule or complex of more than one molecule that affects sAC signal transduction. The present invention contemplates screens for synthetic small molecule agents, chemical compounds, chemical complexes, and salts thereof as well as screens for natural products, such as plant extracts or materials obtained from fermentation broths. Other molecules that can be identified using the screens of the invention include proteins and peptide fragments, peptides, nucleic acids and oligonucleotides (particularly triple-helix-forming oligonucleotides), carbohydrates, phospholipids and other lipid derivatives, steroids and steroid derivatives, prostaglandins and related arachadonic acid derivatives, etc.

In another aspect, synthetic combinatorial libraries (Needels *et al.*, Proc. Natl. Acad. Sci. USA 90:10700-4, 1993; Ohlmeyer *et al.*, Proc. Natl. Acad. Sci. USA 90:10922-10926, 1993; Lam *et al.*, International Patent Publication No. WO 92/00252; Kocis *et al.*, International Patent Publication No. WO 94/28028) and the like can be used to screen for compounds according to the present invention.

Test compounds are screened from large libraries of synthetic or natural compounds. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and

animal extracts are available from *e.g.* Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle *et al.*, Tib Tech, 14:60, 1996).

5 Thus contemplated within the scope of the invention is to use the sAC signaling pathway as an approach to find modulators of sAC activity. For example, inhibiting sAC activity provides a means of preventing, reducing slowing pathological conditions which may result from sAC hyperactivity. For example, because sAC is an oncogene upregulated in human tumors, which might contribute to induction or
10 maintenance of human cancers, defining sAC's signaling cascade will establish whether it could be possible to inhibit sAC activity as a means to slowing or preventing growth of cancers. In this respect pharmacologic and molecular genetic approaches can be used.

 The present invention provides numerous methods for detecting signals, including but not limited to directly detecting cAMP levels. Preferably, gene expression is
15 detected using a reporter gene assay. Alternatively, a downstream element of a signal transduction pathway can be modified to have reporter activity. Reporter genes for use in the invention encode detectable proteins, including, but are by no means limited to, chloramphenicol transferase (CAT), β -galactosidase (β -gal), luciferase, green fluorescent protein, alkaline phosphatase, and other genes that can be detected, *e.g.*, immunologically
20 (by antibody assay).

 The β -galactosidase screen for identifying sAC inhibitors uses a previously described protocol (Tang WJ and AG Gilman 1995) Science 268:1769-72) examining the engineered, soluble form of transmembrane adenylyl cyclases (tmACs). Using the strains and plasmids described therein, we have demonstrated that the truncated, highly active
25 form of sAC, sAC_t, will complement the *cya* mutation in *Escherichia coli*. Basically, *E. coli* harboring a mutated adenylyl cyclase (*cya*) gene will grow WHITE on an indicator plate referred to as MacConkey agar while wild type *E. coli* are usually red. The red colony color indicates the ability to synthesize cAMP and is directly due to a change in the pH of the agar where the colony is growing. The engineered soluble form of tmACs can
30 generate sufficient cAMP to complement the *cya*⁻ mutation and cause bacteria which express it to grow as red colonies. We recently showed that *cya*⁻ bacteria which express sAC_t also form red colonies revealing that it too is able to generate sufficient amounts of

cAMP in bacteria. To identify compounds which specifically inhibit sAC and not tmACs, one could screen a library of compounds against two strains of bacteria, one expressing the engineered soluble form of tmAC and the other expressing sAC_i. Any compound turning sAC_i expressing bacteria white while not affecting the soluble tmAC expressing bacteria, must be selectively preventing sAC from making cAMP.

Other known "target" genes involved in sAC activation can be tested. Chip-based technologies could be employed to determine transcriptional targets specifically involved in sAC transformation. 'Gene chips' can be used to explore the entire panoply of genes regulated by sAC_i. Using this method a specific transcriptional target of sAC can be identified, and the gene's promoter linked to a reporter construct such as Green Fluorescent Protein (GFP), β -galactosidase, luciferase, or chloramphenicol acetyltransferase (CAT). We can then use reporter gene expression as a readout of sAC pathway activation as a primary screen for inhibitors or activators of the sAC pathway. In a specific embodiment, these assays explore the sAC transformation pathway.

15 sAC Inhibitors

Specific inhibitors of sAC include anti-sense and ribozymes, anti-sAC intracellular antibodies, and small molecule inhibitors discovered as set forth herein, including catechol estrogens.

Different putative inhibitors of sAC activity are tested by measuring production of cAMP at different fixed inhibitor concentrations or under other conditions. Various parameters can be analyzed, which include:

- (1) Product inhibition. The products of the cyclization reaction, cAMP and pyrophosphate can be tested for their ability to inhibit cAMP formation.
- (2) Inhibition with alternatively used trinucleotides. sAC's ability to recognize and/or utilize other nucleotides can be tested, for example the nucleotide triphosphates GTP, CTP, TTP, UTP and ITP. If any nucleotide competitively inhibits ATP with millimolar or submillimolar K_i, we will test the possibility that sAC is able to utilize it as substrate to synthesize the corresponding cyclic nucleotide. In all cases, except GTP conversion to cGMP, *in vitro* assay would be used for the corresponding cyclase activity;
- (3) P site ligands and inhibitors of other ATP-utilizing enzymes. P site

inhibitors are adenine nucleotides which inhibit tmAC activity (Johnson, R.A., *et al.*, Methods Enzymol., 1994, 238:56-71). sAC's previously described insensitivity to various P-site inhibitors ((Johnson, R.A., *et al.*, Methods Enzymol., 1994, 238:56-71; Johnson, R.A., *et al.*, Mol. Pharmacol., 1989, 35:681-8) has been confirmed using the P site ligand 2',3'-dideoxyadenosine and the truncated form of sAC. Various P site ligands less effective at inhibiting tmACs may prove useful for sAC, in the hope that they may prove more efficacious at inhibiting sAC. Different physiological (*i.e.* ADP, AMP) and nonphysiological nucleotides (*i.e.* ATP γ S, AMP(NH)P) can also be tested.

Catechol Estrogens The catechol estrogens (2- and 4-hydroxtestradol and 2- and 4-hydroxyestrone) are physiological estrogen derivatives with distinct biological effects compared with their parent compounds. In hypothalamus and pituitary, concentrations of catechol estrogens are at least ten times higher than the parent estrogens (Paul, S.M., *et al.*, Science, 1997, 197:657-9). Contrary to estrogens, catechol estrogens inhibit cAMP accumulation in the hypothalamus (Paul, S.M., *et al.*, Nature, 1977, 266:559-61).

Catechols are labile molecules easily oxidized to quinones, especially in the presence of divalent cations like Mn⁺⁺ (which are present at millimolar concentration in sAC enzyme assays). Quinone formation can be prevented by antioxidants. Therefore, quinones, and not catechols, could be responsible for suppression of cAMP production and the antioxidants could diminish potency of sAC inhibition by preventing the conversion of catechols to quinones.

Various compounds such as 2- and 4-hydroxyestradiol, 2- and 4-hydroxyestrone (Steraloid, Inc.), and not their parent compounds estradiol and estrone, affect sAC activity. This can be shown using purified sAC isoforms in the presence and absence of antioxidants. The mode of inhibition (*i.e.*, competitive or noncompetitive) and the corresponding IC and Ki values can also be determined.

Binding affinities to the distinct sAC isomers for catechols can be determined using tritiated 2-hydroxy estrone (Dupont-NEN). sAC isomers can be incubated with different amounts of [³H]2-hydroxy estrone and unbound ligand is separated by small gel filtration columns. Nonspecific binding is determined by adding excess unlabelled 2-hydroxy estrone. In competition studies, [³H]2-hydroxy estrone will

be competed with other catechols and catechol estrogens. Data will be analyzed using the KELL package of binding analysis programs (Biosoft).

In order to determine whether catechol estrogen itself or an oxidized product is the inhibitor of sAC, isomers are incubated with [³H]2-hydroxy estrone, and unbound catechol estrogen is separated by gel filtration. Protein is dilapidated and the lipids separated using reversed phase HPLC. Oxidized catechol estrogens would have different elution times than catechol estrogens. If this would be the case, the structure of the catechol derivative is determined. A combination of mass spectroscopy, NMR and circular dichroism spectroscopy would be used to characterize the structure of small lipophilic molecules. Even if not physiologically relevant, the structure of the respective bioactive catechol estrogen derivative would be a lead compound to develop drugs specifically inhibiting oncogenic sAC.

Using sAC-inducible and control cell lines, it can be determined whether sAC transforms cells in the presence of commercially available inhibitors which prevent signaling through Ras (N-acetyl-S-farnesyl-L-cysteine, or AFC, which blocks Ras farnesylation), the Raf/MAP kinase pathway (PD-98095 which is selective inhibitor of the MAP kinase kinase, MEK), and the phosphoinositide pathway (LY 294002 or Wortmannin, which are potent and selective inhibitors of PI-3 kinase). We will also test the Ral effector pathway by transfection of a dominant negative Ral mutant (Goi, T., *et al.*, Mol Cell Biol., 1999, 19:1731-41).

sAC Binding Proteins

Regulators of sAC transformation, such as Rap1, can be evaluated in sAC inducible cell lines by using effector protein binding domains which preferentially bind to sAC induced expressed proteins.

A two-hybrid screen can be used to identify sAC interacting proteins. Using sAC as 'bait' (*i.e.*, fused to the GAL4-DNA binding domain) to target libraries consisting of cDNAs generated from NIH 3T3 cells can be screened, which are transformed by sAC and from cell lines derived from colon tumors, which upregulate sAC protein. The screen is performed in the presence and absence of mammalian CAP engineered to contain a nuclear localization signal. Including CAP protein permits identification of any genes interacting exclusively with the sAC-CAP heteromer. Others

have used bridging proteins in two-hybrid selections before; for example, in a directed two-hybrid screen. Ras interaction with MAPKinase kinase required the inclusion of nuclear localized Raf (Van Aelst, L., *et al.*, Proc. Natl. Acad. Sci. USA, 1993, 90:6313-7).

Various reagents for confirming an interaction and identifying the
5 interacting proteins are available. For example, if previously known and studied genes are found, antisera may be used to test for co-immunoprecipitation of sAC and the newly identified potential binding partner. Alternatively, purified protein would permit attempting *in vitro* mixing experiments as described above for CAP or bacterially express GST fusion proteins of the target proteins. If soluble, this will facilitate rapid purification
10 to test *in vitro* binding using purified components.

Potential targets through which sAC transformation can be regulated, include modulating the activity of another, already known, oncogenic signaling pathway. For example, Fos transcription, or more often reporter expression from a Fos gene promoter, can be used as a reliable indicator of transforming pathway activation (Ahn, S.,
15 *et al.*, Mol. Cell Biol., 1998, 18:967-77). Fortunately, there exist a large number of readily available reagents to test whether other known oncogenes or their targets are required for sAC transformation. Using such reagents, sAC transformation can be prevented by blocking signaling through known oncogenic pathways. For example, Ras signaling cascade possesses excellent examples of the various reagents available;
20 including a dominant negative (Feig, L.A., *et al.*, Mol. Cell Biol., 1988, 8:3235-43; Schaap, D., *et al.*, J. Biol. Chem., 1993, 268:20232-6) or effector selective (White, M.A. *et al.*, Cell, 1995, 80:533-41) mutants, and immunological (Bar-Sagi, D., *et al.*, J. Cell Physiol. Suppl., 1987, Suppl:69-73; Kolch, W.A., *et al.*, Oncogene, 1996, 13:1305-14) or pharmacological inhibitors (Choudhury, G.G., *et al.*, Am. J. Physiol., 1997, 273:F931-8;
25 Kohl, N.E., *et al.*, Science, 1993, 260:1934-7; Leftheris, K., *et al.*, J. Med. Chem., 1996, 39:24-36). Furthermore, the analogy with yeast cyclase predicts sAC to be a direct effector of Ras.

To determine whether sAC transformed cells possess a defect in apoptosis, attempts can be made to block sAC transformation by expressing Bcl family members,
30 which promote cell death (*i.e.*, Bcl-X_s, Bax or Bad), or by activation of caspases (*i.e.*, Betulinic Acid), which effect programmed cell death.

To determine whether sAC activity is required for other oncogenes to

transform cells, signaling through sAC should be diminished or blocked. Using for example the ecdysone inducible expression system (Invitrogen) to regulate expression of antisense and dominant negative forms of sAC. Expression of antisense is a widely used technique which often selectively down-regulates protein levels of a target gene (Ho, P.T.,
5 *et al.*, Semin. Oncol., 1997, 24:187-202). Down-regulation of sAC protein in NIH 3T3 may be possible because (1) there do not seem to be any closely related genes in mammals; (2) sAC mRNA levels are low in somatic tissues; and (3) co-transfection of sAC antisense (α sAC) diminished activity of the inducible sAC expression construct. Inducible α sAC can be stably introduced in the EcR/RXR expressing NIH 3T3 cells, and Western Blotting
10 used to reveal the time course of antisense induction required to diminish endogenous sAC protein levels.

Oncogenic Activity of sAC

Another aspect would be to use sAC to test whether other known oncogenes require sAC activity to transform cells. Individual oncogenes are transiently
15 transfected into inducible sAC antisense or dominant negative cell lines to see whether sAC down-regulation (*i.e.*, in the presence of the inducing agent, ponasterone A) hinders the tested oncogene's ability to transform NIH 3T3 cells. A variety of representative oncogenes including, but not limited to, src, myc, jun, bcl2, sis, erbA, erbB, crk, and dominant negative forms of tumor suppressors, such as Rb and p53, can be tested.

20 For example, the 120 kD sAC isoform of sAC is specifically upregulated in human tumors (Fig. 5). This processed molecule contains the potential Leucine Zipper motif identified at approximately 115 kD from the N-terminus. If this Leucine Zipper is functionally important to the 120 kD isoform, then specific inactivation of this isoform may be possible by introducing peptide mimics of its sequence, or a protein fragment
25 which encompasses this sequence. In this way, the 120 kD isoform of sAC can be selectively inhibited without affecting the activity of the 48 kD or other isoforms.

Pharmacologic inhibition of tyrosine and serine/threonine kinases (*i.e.*, herbimycin A or genistein and staurosporine or H-9) and phosphatases (*i.e.*, Benzylphosphonic acid-(AM)₂ and okadaic acid or microcystin), and antioxidants (*i.e.*,
30 CAPE), implicated in cellular transformation, represent candidate pathways for evaluation. Dose-response relationships will be determined for inhibitors which block sAC

transformation. Individual components from biochemical pathways corresponding to any successful inhibitors can be evaluated.

sAC as a Bicarbonate Chemosensor

Multiple mammalian physiological processes are mediated by

5 CO₂/bicarbonate via as yet uncharacterized chemoreceptors. Applicants have found that sAC is directly controlled by bicarbonate/CO₂ levels and therefore sAC may be a physiological chemoreceptor in processes mediated by CO₂/bicarbonate.

Immunoprecipitated sAC activity was stimulated by bicarbonate in the presence of the physiologically relevant substrate Mg²⁺-ATP. These data suggest that sAC is responsible
10 for bicarbonate stimulated cAMP accumulation in tissues such as testis and sperm, and that bicarbonate may be acting directly on sAC enzymatic activity. Because of the intimate relationship between bicarbonate, CO₂ and pH, it is possible that some, if not all, of these signaling processes in physiological systems, including but not limited to those described below, are regulated by bicarbonate modulation of cAMP.

15 Evolutionary precedence for such a signal transducing pathway can be found in cyanobacteria. Using purified recombinant enzyme, applicants have demonstrated that the same adenylyl cyclase which senses light, cyaC, is directly stimulated by bicarbonate. It is believed that this cyanobacterial adenylyl cyclase may be a cellular CO₂/bicarbonate sensor, producing cAMP in direct response to CO₂/bicarbonate to
20 stimulate metabolism.

Physiological processes regulated by bicarbonate/carbon dioxide

Ciliary processes of the eye.

Aqueous humor formation by ocular ciliary processes is dependent on bicarbonate (Kishida *et al.*, 1982; Maren, 1972).

Carbonic anhydrase inhibitors, which affect the balance between CO₂, pH, and bicarbonate
25 ions, can be used to treat glaucoma by decreasing aqueous humor secretion. However, the regulation and mechanism of bicarbonate-dependent secretion are not well understood. There is an established link between ocular aqueous flow and cAMP signal transduction (Caprioli and Sears, Lancet, 1983, 1:958), but how adenylyl cyclase, the enzyme synthesizing cAMP, is regulated remains unclear. Bicarbonate-stimulated adenylyl cyclase
30 activity has been found in ciliary processes (Mittag *et al.* 1993) suggesting that a distinct

form of mammalian adenylyl cyclase may be present in these secretory epithelia which may provide the link between bicarbonate, cAMP signaling, and aqueous humor formation., i.e. bicarbonate-dependent increase in cAMP signaling leading to aqueous humor formation. Based on the discovery by applicants that sAC is stimulated by bicarbonate, it is believed that sAC may be the bicarbonate sensing receptor mediating changes in cAMP metabolism and may affect fluid secretion in secretory epithelia. Thus sAC may be a target for alternate therapy or treatment for glaucoma. Accordingly, the present invention provides a method of treating glaucoma by reducing aqueous humor formation stimulated by cAMP signaling by administering to a subject afflicted with glaucoma a modulator of sAC activity in an amount effective to reduce or inhibit sAC activity to decrease the production of cAMP. It is contemplated that administration of a sAC activity modulator or inhibitor can be effected through methods known to those of skill in the art for treating glaucoma or other eye conditions, such as in eye drops, in suspension, emulsion and the like, and may be administered daily, however the timing of administrations or applications will take into account the severity of the condition, the age of the subject, etc. The sAC modulator or inhibitor may be used optionally in conjunction with one or more of a variety of agents suitable for the preparation of different formulations which will be selected in accordance with the type of formulation and route of administration desired. Included are buffers salts, preservatives, and the like, and optionally may further include other biologically active agents.

Choroid plexus of the brain Cerebrospinal fluid formation by the choroid plexus is also dependent on bicarbonate (Maren, Am.J.Physiol., 1972, 222:885-899). In choroid plexus transport systems, carbonic anhydrase inhibitors decrease fluid secretion (Maren, Annu. Rev. Physiol., 1988, 50:695-717). Others have reported the existence of a bicarbonate-stimulated adenylyl cyclase activity in the choroid plexus, but not in the cerebral cortex (Mittag *et al.*, 1993). sAC may be the bicarbonate sensing receptor mediating changes in cAMP metabolism and may affect fluid secretion in secretory epithelia. Accordingly, sAC may be a target for therapy or treatment of conditions such as hydrocephaly by using inhibitors of sAC activity.

Breathing. Partial CO₂ pressure (P_{CO2}) is a critical value in determining the

rate of ventilation (Johnson, 1998). An increase of P_{CO_2} increases breathing frequency, and
a

decrease of P_{CO_2} decreases breathing frequency. These rate changes are mediated by
peripheral and central chemoreceptors which monitor changes in arterial blood gases.

- 5 Peripheral arterial chemoreceptors sense changes in P_{O_2} and P_{CO_2} in the carotid and aortic
bodies, and they detect alterations in pH in the carotid bodies. The central chemoreceptive
area at the ventral surface of the medulla senses changes only in arterial P_{CO_2} . The
molecular nature of each of these types of chemoreceptors is unknown. Based on the
discovery by applicants that sAC is stimulated by bicarbonate, it is possible that sAC may
10 function as the bicarbonate chemoreceptor.

- The present invention provides a method for altering breathing rate or
frequency by providing sAC as a target for drug or other therapeutic agents to modulate
sAC activity. Agents which modify sAC's ability to sense bicarbonate make it possible to
alter the equilibrium between CO_2 /bicarbonate. In this manner, in situations where oxygen
15 absorption is compromised, e.g., high altitude, stress, marathon running, athletic training,
breathing rate or frequency may be advantageously facilitated and physical activity
sustained.

- Blood flow.** Blood flow is tightly coupled to tissue metabolism (Johnson,
1998). Carbon dioxide, protons, and adenosine relax smooth muscle and act as
20 vasodilators. For example, cerebral arterioles dilate in response to increases in metabolic
activity of the brain. Cerebral arterioles are exquisitely sensitive to the vasodilatory action
of P_{CO_2} . However, the molecular nature of the vascular P_{CO_2} receptor is unknown. sAC
may be the CO_2 sensor mediating this vasodilatory response to metabolism.

Bicarbonate regulation by the kidney

- 25 Serum bicarbonate concentration is tightly regulated by the kidney to be 22-
26 mM. The kidney reabsorbs all bicarbonate filtered at the glomerulus and generates new
bicarbonate by excreting 'titratable acidity' (H^+ combined with urine buffers such as
 HPO_4^{2-} and SO_4^{2-}) and ammonium ions. The proximal tubules "reabsorb" 95% of the
filtered bicarbonate (Johnson, 1998). Dependent on the body's acid-base balance, the
30 distal convoluted tubules and the collecting ducts can absorb or secrete bicarbonate
(Johnson, 1998); however, it is unknown how the kidney senses serum bicarbonate

concentration to determine which is appropriate. A bicarbonate-stimulated adenylyl cyclase activity was reported in kidney medulla and cortex (Mittag *et al.*, 1993). Based on the discovery by applicants that sAC is stimulated by bicarbonate, it is possible that sAC may function as a renal bicarbonate sensor.

5 Accordingly, the present invention provides a means of modulating serum bicarbonate concentration by modulating sAC activity, as described herein, in the kidney, thereby altering the kidney's mechanism for bicarbonate regulation.

Biosensors

 The discovery that bicarbonate stimulates sAC activity also provides a
10 means for detecting and quantifying bicarbonate in a body fluid using sAC as a sensor. The monitoring of physiological bicarbonate levels is applicable in emergency situations, intensive care, during surgery neonatal care where monitoring of the blood gases is critical to avoid excessive or subnormal levels of carbon dioxide or bicarbonate resulting in metabolic or respiratory acidosis or alkalosis. Blood gas level measurements can be used
15 in non-emergency situations to monitor the ratio of CO₂/bicarbonate in order to manipulate the ratio to alter breathing rate and frequency, for example for high altitude sickness, marathon runners, and endurance athletes.

 Devices which measure blood chemistry (pH, CO₂, PO₂) are well known in the art. Current methods generally used in biosensor technology include a variety of
20 combinations of biological elements and transducing elements such as colorimetric, optical, potentiometric and the like (Biosensors Turner Oxford University Press, New York 1987).

 sAC may be used as a sensor in its capacity as an enzyme to determine physiological bicarbonate levels. These methods include utilizing reporter genes, such as
25 β-galactosidase, described above, or immunological detection. sAC stimulation by bicarbonate can be detected using the methods described above for determining sAC activation. Alternatively, since in the presence of bicarbonate sAC is activated and cAMP is generated, the invention also includes quantifying bicarbonate levels by directly detecting cAMP production. The amount of cAMP detected is directly correlatable to the
30 amount of bicarbonate in the sample. Methods for measuring cAMP production are well known to those of skill in the art. A system for detection that can be used in conjunction

with sAC as a sensor molecule suitable for the present invention includes one based on the occurrence of an enzymatic reaction, detected by energy transfer, wherein the reaction is dependent on the presence of cAMP (see, e.g., U.S. Pat. No. 5,439,797).

sAC as a Regulator of Male Fertility

5 Sperm functions thought to be mediated by cAMP include sperm maturation, mobility and the acrosome reaction. Less clear is the molecular basis of sperm capacitation, the series of events that provide sperm with the ability to fertilize an egg. Ca^{2+} and HCO_3^- are linked to the regulation of sperm cAMP concentration by their effect on the stimulation of adenylyl cyclase activity. (Visconti P.E. et al, Journal of Andrology, 10 1998, 19(2): 242-248). Based in part on the discovery that sAC activity is stimulated by treatment with bicarbonate, the present invention provides a mechanism for mediating sperm capacitation.

 Thus the present invention provides a method of inhibiting fertilization of an ovum by inhibiting a soluble adenylyl cyclase activity in sperm. Soluble adenylyl 15 cyclase activity can be inhibited, for example, by contacting sperm with a soluble adenylyl cyclase inhibitor, such that sAC activity is reduced or eliminated resulting in decreased or inhibited capacitation of sperm. It is contemplated that a sAC inhibitor could be provided in a formulation which will contact the sperm at various locations in the capacitation pathway.

20 Soluble adenylyl cyclase activity can also be regulated in order to facilitate or increase sperm capacitation. Facilitating sperm capacitation is particularly advantageous, for example, in the process of *in vitro* fertilization (IVF). IVF sperms are separated from seminal fluid and exposed to a proteinaceous cocktail which induces the capacitation reaction in the isolated spermatosa. By adding a factor that activates sAC, it 25 is contemplated that the success of IVF may be improved in those instances which the sperm are in any way defective.

sAC as a Regulator of Insulin Secretion

 Data indicate soluble adenylyl cyclase (sAC) may be regulated *in vivo* via changes in intracellular pH, therefore sAC may be involved in glucose regulation of 30 insulin release from pancreatic islet cells. In islet cells, glucose regulated insulin release is

a cAMP dependent process, but it is not known how glucose alters cAMP and the glucose sensor has not been identified. Glucose uptake is known to alter intracellular pH, but the connection to insulin release essentially ends there. sAC is regulated by bicarbonate and bicarbonate levels directly reflect intracellular pH, suggesting it could be the link between islet cells sensing glucose concentration and releasing insulin.

Polypeptide and Gene Therapy

The methods of the invention are useful in treating diseases characterized by a deficiency or an inappropriately high level of activity of cells. The invention provides methods of modulating the sAC signaling pathway.

Peptides may be administered to the patient intravenously in a pharmaceutically acceptable carrier such as physiological saline. Standard methods for intracellular delivery of peptides can be used, e.g. delivery via liposomes. Such methods are well known to those of ordinary skill in the art. The formulations of this invention are useful for parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal.

Therapeutic administration of a polypeptide intracellularly can also be accomplished using gene therapy, wherein a nucleic acid which includes a promoter operatively linked to a sequence encoding a heterologous polypeptide is used to generate high-level expression of the polypeptide in cells transfected with the nucleic acid. DNA or isolated nucleic acid encoding peptides of the invention may be introduced into cells of the patient by standard vectors and/or gene delivery systems. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, and adenoviruses, among others. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal, e.g., physiological saline. A therapeutically effective amount is an amount of the nucleic acid of the invention which is capable of producing a medically desirable result in a treated animal.

As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages for the compounds of the invention

will vary, but a preferred dosage for intravenous administration is from approximately 10^6 to 10^{22} copies of the nucleic acid molecule in the case of gene therapy.

Diagnostic Applications

5 The present invention encompasses compositions, methods, and kits useful in clinical settings for the qualitative or quantitative diagnosis of conditions arising from sAC activation. These applications typically utilize nucleic acids, peptides/polypeptides, or antibodies specific for sAC components. The methods may also be used to detect sAC specific cell lines and to detect sAC positive cells in a patient, in particular a human patient.

10 **Antibody-based diagnostic methods**

The invention provides methods for detecting sAC antigenic components in a biological sample, which methods comprise the steps of: (i) contacting a sample suspected to contain sAC antigenic component with an antibody specific for a sAC antigen, extracellular or intracellular, under conditions in which a stable antigen-antibody complex can form between the antibody and the antigenic components in the sample; and
15 (ii) detecting any antigen-antibody complex formed in step (i) using any suitable means known in the art, wherein the detection of a complex indicates the presence of sAC antigenic components in the sample. It will be understood that assays that utilize antibodies directed against sequences previously unidentified, or previously unidentified as being sAC-specific, which sequences are disclosed herein, are within the scope of the
20 invention.

Many immunoassay formats are known in the art, and the particular format used is determined by the desired application. An immunoassay may use, for example, a monoclonal antibody directed against a single sAC epitope, a combination of monoclonal
25 antibodies directed against different epitopes of a single sAC antigenic component, monoclonal antibodies directed towards epitopes of different antigens, polyclonal antibodies directed towards the same antigen, or polyclonal antibodies directed towards different antigens. Protocols may also, for example, use solid supports, or may involve immunoprecipitation.

30 Typically, immunoassays use either a labeled antibody or a labeled

antigenic component (e.g., that competes with the antigen in the sample for binding to the antibody). Suitable labels include without limitation enzyme-based, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays that amplify the signals from the probe are also known, such as for example, those that utilize biotin and avidin, and
5 enzyme-labeled immunoassays, such as ELISA assays.

Kits suitable for antibody-based diagnostic applications typically include one or more of the following components:

(i) Anti-sAC antibodies: The antibodies may be pre-labeled; alternatively, the antibody may be unlabeled and the ingredients for labeling may be included in the kit
10 in separate containers, or a secondary, labeled antibody is provided; and

(ii) Reaction components: The kit may also contain other suitably packaged reagents and materials needed for the particular immunoassay protocol, including solid-phase matrices, if applicable, and standards.

The kits referred to above may include instructions for conducting the test.
15 Furthermore, in preferred embodiments, the diagnostic kits are adaptable to high-throughput and/or automated operation.

Nucleic-Acid-Based Diagnostic Methods

The invention provides methods for detecting sAC-derived nucleic acids in a sample, such as a biological sample, which methods comprise the steps of: (i) contacting
20 a sample suspected of containing sAC-derived nucleic acid with one or more sAC-derived nucleic acid probes under conditions in which hybrids can form between any of the probes and sAC nucleic acid in the sample; and (ii) detecting any hybrids formed in step (i) using any suitable means known in the art, wherein the detection of hybrids indicates the presence of sAC nucleic acid in the sample.

25 sAC-specific nucleic acids useful as probes in diagnostic methods include oligonucleotides at least about 8 nucleotides in length, preferably at least about 12 nucleotides in length, and most preferably at least about 15-20 nucleotides in length, that specifically hybridize the sAC gene.

A sample to be analyzed, such as for example, a biological sample or an
30 environmental sample, may be contacted directly with the nucleic acid probes. Alternatively, the sample may be treated to extract the nucleic acids contained therein. It

will be understood that the particular method used to extract DNA will depend on the nature of the biological sample. The resulting nucleic acid from the sample may be subjected to gel electrophoresis or other size separation techniques, or, the nucleic acid sample may be immobilized on an appropriate solid matrix without separation or used for
5 PCR.

PCR based diagnostic kits are also contemplated and are encompassed by the invention.

Kits suitable for nucleic acid-based diagnostic applications typically include the following components:

- 10 (i) Probe DNA: The probe DNA may be pre-labeled; alternatively, the probe DNA may be unlabeled and the ingredients for labeling may be included in the kit in separate containers; and
- (ii) Hybridization reagents: The kit may also contain other suitably packaged reagents and materials needed for the particular hybridization protocol, including
15 solid-phase matrices, if applicable, and standards.

EXAMPLES

The present invention will be better understood by reference to the following examples, which illustrate embodiments of the invention but are not limiting.

Example 1: Purification, Molecular Cloning and Functional Expression of sAC.

Materials and Methods

20 **Cyclase Assay.** *In vitro* adenylyl cyclase assay was performed as described previously (Levin, L.R. et al, Cell, 1992, 68:479-489; Levin LR et al., J. Biol., Chem., 1995, 270:7573-7579), except that the standard assay conditions for sAC activity included 5 mM MnCl₂ or MgCl₂ as indicated and contained 5 mM (α -³²P) ATP (specific
25 activity = about 4×10^4 cpm/nmol).

sAC Purification. sAC (approximately 3 μ g) was purified from 950 rat testes by sequential column chromatography by using the following scheme (i) Frozen rat testes (950) (Pel-Freez Biologicals) (in batches consisting of 50 testes) were homogenized and sonicated in 20 mM Tris-HCl, pH 7.5, in the presence of DTT and proteinase
30 inhibitors. After debris and nuclei were removed by low-speed centrifugation (3000 x g for

10 min), a high-speed supernatant ($>100,000 \times g$ for 60 min) was prepared. (ii) Total cytosolic protein (52 g) was dialyzed and separated (as 19 equal portions consisting of 50 testes each) over DE-52 cellulose anion exchange columns (Whatman; 80 ml bed volume; 20 mM Tris-HCl, pH 7.5) by using a linear NaCl gradient. All sAC activity bound and
5 eluted as one peak between 0.15 to 0.2 M NaCl. (iii) sAC activity recovered from DE-52 (4 g protein divided into 11 aliquots of 8 mls each) was separated by using an Ultrogel AcA54 gel filtration column (LKB; 4 x 100 cm/20 mM Tris-HCl, pH 7.5; flow rate 1.0 ml/min). Most sAC activity reproducibly eluted in a single peak with an apparent mass of 50–60 kDa. (iv) All sAC peak fractions from AcA54 gel filtration were pooled (1 g
10 protein) and applied to a reactive Red 120-Agarose column (Sigma; 50 ml bed volume; 20 mM Tris-HCl, pH 7.5; linear gradient 0.1–1.0 M NaCl; flow rate 2 ml/min; 600 ml total). Cyclase activity eluted between 0.45 and 0.55 M NaCl. (v) Active fractions (66 mg protein) were pooled, dialyzed, and applied to a Source Q anion exchange column (Pharmacia; 15 ml bed volume; 20 mM Tris-HCl, pH 7.5; linear gradient 0–0.3 M NaCl;
15 flow rate 0.5 ml/min; 150 ml total). sAC activity eluted between 0.10 and 0.15 M NaCl. (vi) Active fractions (9 mg protein) were pooled, concentrated, and applied to a reactive Green 19-Agarose column (Sigma; 9 ml bed volume; 20 mM Tris-HCl, pH 7.5; linear gradient 0.1–1.0 M NaCl; flow rate 0.6 ml/min; 80 ml total). Cyclase activity eluted between 0.40 and 0.50 M NaCl. (vii) Active fractions (1.8 mg) were pooled, concentrated,
20 and loaded onto a semipreparative HydroCell QA 1000 HPLC anion exchange column (Biochrom, Terre Haute, IN; 50 x 4.6 mm; 20 mM Tris-HCl, pH 7.4; linear gradient 0–0.3 M NaCl over 30 min; flow rate 2 ml/min). Cyclase activity eluted between 0.07 and 0.10 M NaCl. (viii) Active fractions (0.6 mg) were pooled and loaded onto an analytical QA 1,000 HPLC anion exchange column (HydroCell Biochrom; 150 x 2.3 mm; 20 mM Tris-
25 HCl, pH 6.8; linear gradient 0–0.1 M NaCl over 25 min; flow rate 1.5 ml/min; 0.5 ml/fraction). Cyclase activity eluted between 0.04 and 0.06 M NaCl. (ix) Active protein fractions were separated on SDS/PAGE, stained with Coomassie Blue G-250, and the 48- and 62-kDa bands were excised. Protein sequence data were obtained at the Rockefeller University Protein/DNA Technology Centers (Fernandez J. et al. (1994) Anal. Biochem.
30 218: 112-117. Fernandez J. et al. (1992) Anal. Biochem. 201: 255-264) from approximately 3 μ g of recovered 48-kDa protein. Table I provides protein and activity yields recovered during the purification procedure.

Table 1: Purification of sAC from 950 rats

| AC Activity | | | | |
|---------------------------------|----------------|-------------------------|--|------------------------|
| | Protein, mg | Total Units nmol/min | Specific activity nmol/min/mg x 100 | Fold enrichmen t |
| Cytosol | 51,900 | 2,400 | 4.6 | 1 |
| Preparative DE-52 | 4,015 | 3,000 | 75 | 16 |
| 5 Gel filtration AcA54 | 1,074 | 2,100 | 200 | 43 |
| Reactive Red | 66 | 1,200 | 1,800 | 390 |
| Source Q | 8.8 | 1,100 | 12,500 | 2,700 |
| Reactive Green | 1.8 | 380 | 21,000 | 4,600 |
| 10 Semipreparative QA, pH = 7.4 | 0.6 | 310 | 52,000 | 11,300 |
| Analytical QA, pH = 6.8 | | | | |
| Fraction #18 | 0.003 | 90 | 3,000,000 | 650,000 |
| Fraction #19 | 0.010 | 92 | 920,000 | 200,000 |

15 Protein concentrations determined by OD280. Units refer to nmol of cAMP formed per minute. Fold enrichment represents specific activity after each step compared to the starting specific activity.

Results. These results confirmed the presence of Mn²⁺-dependent AC activity in cytosolic extracts from rat testis. The soluble enzymatic activity detected was unresponsive to either forskolin or the nonhydrolyzable GTP analogue, GTP γ S, two
 20 general tmAC activators, and it displayed a *K_m* for ATP of 1.2 mM in the presence of MnCl₂. sAC activity was purified using a combination of classical chromatographic methods shown in Table 1 and identified a 48-kDa candidate protein band by SDS-PAGE. The final chromatographic separation (step *viii*; analytical QA 1,000 HPLC) achieved greater than 60-fold enrichment (Table 1, fraction #18) even though it used the identical
 25 QA anion exchange matrix as the HPLC column preceding it (step *vii*; semipreparative QA 1,000 HPLC). By varying the buffer pH (pH = 7.4 for the semipreparative QA vs. pH = 6.8 for the analytical QA), sAC activity eluted before the majority of contaminating proteins during this final chromatographic separation. A silver-stained 12% SDS/PAGE gel of the active fractions from the final chromatographic step of the purification revealed two

protein bands (of approximately 48 and 62 kDa) whose intensities coeluted with enzyme activity. During pilot purification studies, analytical gel filtration of partially purified cytosol predicted sAC to be 45–55 kDa, suggesting the more likely candidate was the 48-kDa protein. The limited amino acid sequence information obtained from the 62-kDa polypeptide revealed it was completely unrelated to the sAC gene.

Molecular Cloning. Fully degenerate oligonucleotide primers designed to recognize the amino acid sequences of peptides derived from the 48-kDa purified polypeptide (Fig. 1, double underlined) were synthesized for use in PCR amplification of rat testis first-strand cDNA. A 1-kilobase (kb) PCR fragment was generated that had a single ORF extending throughout its length and that contained sequences corresponding to all three peptides. This 1-kb PCR fragment was used as probe to screen a rat testis cDNA library constructed in our laboratory (λ Zap, Stratagene). Four overlapping cDNA clones were obtained from over 7.5×10^5 plaques. Among these, one represented a complete full-length cDNA clone. The nucleotide sequence of the full-length cDNA was determined on both strands by dye termination-automated DNA sequencing (Cornell University DNA sequencing Core Facility, Ithaca, NY) and confirmed by comparison to single-stranded sequence determined from at least one other independent cDNA clone. Sequence and database searching was performed on-line by means of BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) or PSORT II (<http://yipsort.nibb.ac.jp:8800/>).

Results. The sAC gene identified in rat, mouse, and human encodes a cytosolic form of adenylyl cyclase that is distinct from the previously characterized mammalian tmACs. Not only is sAC not a trans-membrane protein, but its catalytic domains are more closely related to the catalytic portions of bacterial ACs than they are to the catalytic domains of any other eukaryotic cyclase. In contrast, the mammalian tmACs, which are distantly related to these bacterial ACs and sAC, more closely resemble other invertebrate (*Drosophila*) and lower eukaryotic (*Dictyostelium*) Acs. The amino acid sequences of three tryptic peptides derived from the 48-kDa candidate polypeptide were not present in the databases of known proteins, indicating it represented a novel protein. The cDNA encoding this polypeptide was isolated by PCR followed by screening a rat testis cDNA library. All isolated cDNAs appeared to derive from one transcript whose nucleotide sequence revealed a single long ORF predicting a protein of 187 kDa (Fig. 1), which is significantly larger than the size (48 kDa) estimated by SDS/PAGE of the

purified sAC protein. The peptides (SEQ ID NOS: 3-5) derived from the 48-kDa purified polypeptide reside completely within the amino terminal portion of the full-length protein, suggesting the purified polypeptide represents a proteolytically processed active form of the protein.

5 Comparison of this ORF with known protein sequences revealed two distinct regions of the putative sAC protein that display significant amino acid homology to various adenylyl cyclase catalytic domains (Fig. 1, bold type). Both sAC domains, C1 and C2, reside within its amino terminal 50 kDa and are therefore likely to be contained within the purified catalytically active processed form. The most closely related protein
10 sequences in GenBank are the catalytic domains from a number of different cyanobacterial (*Anabaena spirulensis* cyaB1, cyaB2, and cyaA; and *Spirulina platensis* cyaC) and myxobacterial (*Stigmatella aurantiaca* cyaA and cyaB) adenylyl cyclases. These species have multiple AC genes with each isoform having a single catalytic domain. Interestingly, the catalytic domain of one AC isoform in each bacterial species is more similar to C1,
15 whereas the catalytic domain of a second isoform from that species more closely resembles C2. This and the fact that C1 and C2 are not very similar to each other may suggest that during its evolution, mammalian sAC resulted from a fusion of distinct bacterial proteins rather than through duplication of a single catalytic domain.

 There is also significant similarity between the two presumptive sAC
20 catalytic domains and other AC catalytic domains. Alignment of C1 and C2 with the catalytic domains from related bacterial ACs, yeast ACs, *Dictyostelium* tmACs, and representative mammalian tmACs reveals that sAC C1 and C2 are more closely related to the catalytic portions of bacterial ACs than to the catalytic domains of any other cyclases. This similarity provides an evolutionary link between bacterial and mammalian signaling
25 systems and suggests that the C1 and C2 catalytic domains in mammalian sAC are likely to have evolved independently from those in eukaryotic tmACs (C1_a and C2_a).

 The C-terminal portion beyond the AC homologous regions revealed no significant homology to any known protein in the databases, and the hydropathy profile of the full-length protein indicated no obvious potential transmembrane-spanning domains.
30 Sequences that could represent a nucleotide-binding P Loop (Fig. 1, dotted underline) or that could form a leucine zipper interacting domain (Fig. 1, single underline) were detected within the region unrelated to the AC catalytic domains.

Hybridizations. Southern and Northern blots were probed with random-primed [³²P]dCTP-labeled 1-kb PCR-generated fragment 483-1536 under standard conditions. Southern blot was hybridized at 65°C overnight and washed three times in 1X SSC (0.15 M sodium chloride/0.015 M sodium citrate, pH 7/0.1% SDS) for 15 minutes at 55°C (low stringency) or three times in 0.53 SSC/0.1% SDS for 15 minutes at 65°C (high stringency).

For Southern blots, 10 µg rat genomic DNA was digested with BamHI, EcoRI (E), HindIII (H), and XhoI (X) probed at high or low stringency by using the 1-kb PCR-generated sAC fragment containing both presumptive catalytic domains. Rat multiple tissue Northern blot (CLONTECH) representing approximately 2 µg poly-A⁺ RNA from testis (T), kidney (K), skeletal muscle (Sk), liver (Li), lung (Lu), spleen (S), brain (B), and heart (H) probed with 1-kb PCR-generated sAC fragment or with actin control. The sAC transcript is approximately 5.3 kb and, in most tissues, actin is approximately 2.0 kb.

Results Southern hybridization to rat genomic DNA, along with numerous database searches, indicated the presumptive sAC gene does not appear to be the progenitor of a gene family of sAC-like molecules in mammals. A sAC coding sequence probe 483-1536 hybridized at high and low stringency to parallel rat genomic Southern blots recognized identical genomic fragments, indicating the lack of closely related sequences in the genome.

sAC mRNA is most abundant in male germ cells, but it is widely expressed. Northern and RNA *in situ* analysis of expression in testis revealed high levels of sAC message exclusively in germ cells beginning in pachytene spermatocytes and accumulating to highest levels in round spermatids. This high level expression seems to reflect the previously determined biochemical restriction to testis, and suggests that sAC plays a prominent role in male reproductive physiology.

Additionally, we have identified the corresponding human and mouse sAC genes by database searches and reverse transcription-polymerase chain reaction (RT-PCR), respectively. The human sAC locus has been sequenced as part of the Genome Project. It is encoded by more than 30 exons that are spread across two overlapping P1-derived artificial chromosome (PAC) clones mapping to 1q24.

Using RT-PCR, sAC message can be readily detected in almost all tissues

examined; RT-PCR products readily seen by ethidium bromide staining can be generated following thirty cycles of amplification from oligo-dT primed first strand cDNA using two sets of non-overlapping primers. The specificity of these PCR products was confirmed by Southern blotting. Its widespread expression, along with its conservation with bacterial
5 signaling systems, suggest that sAC plays an additional, fundamental physiological role in all cells.

RT-PCR amplification of sAC revealed that it is widely expressed at low levels. Non-overlapping primer pairs used for RT-PCR of sAC cDNA amplified an 881 bp DNA fragment for primers 1 and 2 (SEQ ID NO: 6 CGAGCAGCTGGTGGAGATCC and
10 SEQ ID NO: 7 GCGTGAGTGATCTCGTCAGGGGC), respectively, and a 748 bp DNA fragment for primer pairs 3 and 4 (SEQ ID NO: 8 CCTGCTTCTCCCTGCTGTG and SEQ ID NO: 9 GCAGGAGTAAAGTCCCAGG, respectively). Ethidium bromide stained agarose gel of RT-PCR products from brain, ovary, liver, lung, spleen, heart, embryo, thymus, testis.

15 **Heterologous Expression.** To confirm that the gene we isolated encoded an adenylyl cyclase, we heterologously expressed the full-length cDNA in HEK293 cells. The full-length and truncated sAC cDNAs were expressed from the library vector (pBK-CMV) under the control of the cytomegalovirus promoter after deletion of the intervening bacterial promoter sequences (as an *NheI* - *SpeI* fragment). The truncated sAC cDNA
20 (sAC₁) represented a library clone that was missing an exon. The resultant protein shifted the reading frame after valine 469 (Fig. 1, underline), introducing two incorrect amino acids (serine and cysteine) followed by a stop codon. Expression constructs were transiently introduced into HEK293 by lipofectamine - mediated transfection (Life Technologies, Grand Island, NY). One or two days after transfection, cells were harvested,
25 resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5/1 mM EDTA/1 mM DTT/0.1 mg/ml Leupeptin/1 mM phenylmethylsulfonyl fluoride) and disrupted by microtip probe sonication on ice. Whole-cell sonicates were ultramicrocentrifuged at >100,000 x g for 10 minutes. Supernatants were cleared by a second centrifugation to yield 'cytosolic' extracts. Pellets were resuspended in lysis buffer by passage through a 27.5-gauge needle to
30 generate 'particulate fraction.

Results Vector-transfected cells had no detectable soluble and very little unstimulated particulate AC activities. In contrast, cells transfected with the full-length

sAC gene displayed substantial Mn 2+ -dependent soluble AC activity (approximately 85 pmol cAMP/min/mg). Mn 2+-dependent activity was also elevated in the particulate fraction from transfected cell sonicates.

Because the sAC polypeptide purified from rat testis was approximately 48 kDa, we sought to determine whether the truncated version of sAC retained catalytic activity. The expression construct consisting of the amino terminal 53 kDa of sAC encompassing both presumptive catalytic domains was fully active, displaying an extremely high level of Mn 2+-dependent soluble AC activity (approximately 3500 pmol cAMP/min/mg). Therefore, the sAC purified from rat testis cytosol very likely represents a proteolytically processed form resembling this N-terminal truncation. The extremely high level of cytosolic activity in cells expressing the truncated form compared with those expressing the full-length protein is consistent with processing being required for catalytic activity; the activity in cells transfected with full-length sAC may be limited by the availability of activating enzymes.

We also examined whether heterologously expressed sAC responded to known stimulators of tmAC activity G protein and forskolin. When assayed in whole-cell sonicates from transfected HEK293 cells, which should contain the full complement of endogenous G proteins, both full-length and truncated forms of sAC were completely insensitive to forskolin and to the nonspecific G protein activator, GTP γ S respectively.

The N-terminal 50 kDa of sAC is sufficient for enzymatic activity and approximately corresponds to the size of the protein purified from rat testis cytosol. Because all the cDNAs isolated from rat testis fell into a single class encoding the 187-kDa polypeptide, the 48-kDa purified protein should result from posttranslational cleavage. Truncating the sAC gene increased cyclase activity 10–20 fold in tissue culture cells, suggesting that the shorter molecule approximated an activated form. The activity directed in cells transfected with the full length cDNA may result from a cleaved molecule.

Production of anti-sAC Antisera We generated three anti-sAC antisera. An antiserum for the N-terminal peptide SARRQELQDRAIVK (SEQ ID NO:10) was obtained. This peptide was produced as a MAP, or multigen antigenic peptide, by Research Genetics, Inc. and used to immunize rabbits.

The antigen for the N-terminal Catalytic Domain (α C1-C2) antisera is from the Methionine at position number 1 to the Valine at position #469. This antigen was used by HTI, Inc. to immunize rabbits.

5 The antigen for the C-Terminal (α C-Term) antisera is from the Isoleucine at position #1409 to the end of the protein coding sequence (Amino acid #1608). This antigen was used by HTI, Inc. to immunize chickens. We prepared rabbit antiserum against the first 15 N-terminal amino acids (α N-term), which are shared between mouse and rat; rabbit antiserum against the catalytic region (α C1-C2); and chicken antiserum against the C-terminus (α C-term). We constructed, expressed and purified two His-
10 tagged fusion protein corresponding to the 50 kD rat N-terminus and the 20kD rat C-terminus of sAC for use as immunogens. The generated antisera were affinity purified by negative selection against the shared His-tag and positive selection against the specific immunogen. We raised the α C-term antisera in chickens for use in double labeling studies together with rabbit anti N-terminal or catalytic antisera.

15 The affinity purified sAC-specific antisera raised against either the catalytic N-terminal 50 kD (α C1-C2) (Fig. 2A), the C-terminal 25 kD (α C-term), or the 14 N-terminal amino acids (α N-term), recognize only sAC proteins in whole cell lysates of HiFive cells infected with recombinant baculovirus expressing Truncated (T) or Full-length (F) sAC. The sAC specific bands are not recognized by preimmune serum.

20 **Immunoprecipitation of sAC Activity** Supernatants from sAC_t transfected HEK293 cells were incubated with (0, 0.3, 0.6, 1.2, 2.5 and 5 μ l) anti-sAC –terminal antibody and precipitated with 50 μ l of Protein A beads (BIO-RAD). Immunoprecipitates were assayed using the soluble adenylyl cyclase assay. sAC transfected HEK293 cell supernatants also were incubated with 5 μ l of anti-sAC –terminal
25 antibody. Antibody-antigen complex was precipitated with 12.5, 25 or 50 μ l of Protein A beads using standard methods. The α C1-C2 antisera (crude or affinity purified) immunoprecipitated heterologously expressed sAC activity.

sAC protein is processed into multiple isoforms Purified sAC activity from rat testis resided in a 48 kD polypeptide, yet the encoding cDNA predicted a 187 kD
30 protein. Western analysis of mouse germ cells from different stages of spermatogenesis using the affinity purified sAC-specific antibody revealed the presence of multiple, developmentally regulated isoforms of sAC. Using purified preparations of mouse primary

spermatocytes (pre-meiotic germ cells), round spermatids (post-meiotic germ cells undergoing spermiogenesis or differentiation into mature spermatozoa), and epididymal sperm (spermatozoa maturing as they transit through the epididymis), we detected five distinct immunoreactive bands, of 190 kD, 150 kD, 120 kD, 48 kD, and 45 kD (Table 2).

Table 2
Immunoreactive bands recognized by sAC specific antisera

| Protein bands | α N-term | α Catalytic (C1-C2) | α C-term |
|----------------|-----------------|-------------------------------|-----------------|
| 190 kD | + | + | + |
| 150 kD triplet | - | + | - |
| 120 kD | - | + | - |
| 48 kD | - | + | - |
| 45 kD | + | + | - |

All bands were recognized by the anti C1-C2 antiserum. The 190 kD species corresponds to full-length, native sAC protein; it is recognized by all three sAC-specific antisera. As there are no alternatively spliced transcripts from the sAC gene, we assume that the remaining bands correspond to post-translationally processed isoforms. The 45 kD form is additionally recognized by α N-terminal antiserum. The 48 kD form corresponds to the sAC activity we purified. The 120 kD species is only recognized by the anti C1-C2 antiserum. While both the 48 kD and the 45 kD isoform do not appear to vary during spermatogenesis, the 120 kD sAC isoform seems to be developmentally regulated. We believe this isoform is specifically generated during final sperm maturation. Interestingly, because of its size and the fact that it is recognized by α C1-C2 but not by α C-term, this isoform would include both the potential nucleotide-binding P loop at 55 kD and the leucine zipper interacting domain at 115 kD. Additionally, there is a triplet of specific protein bands at approximately 150 kD detected only in pachytene spermatocytes (PS) and round spermatids (RS) which could correspond to distinct proteolytic processing intermediates. Fig. 3 is a photograph of the Western blot.

Example 2: sAC in bicarbonate sensing tissues

Experiments were performed to determine whether native sAC was present in tissues known to regulate bicarbonate concentrations and have been found to contain bicarbonate stimulated adenylyl cyclase activity.

Samples of testis, sperm, kidney, and choroid plexus were tested against anti-sAC antibody in a Western blot, shown in Figure 4a. The lanes contained the following amount of sample: testis (30 μ g), sperm (5 μ g), kidney (50 μ g), and choroid plexus (50 μ g). sAC was detected in all these tissues.

Rat testis were tested for sAC activity by immunoassay.

Immunoprecipitates from rat testis cytosol were tested using either pre-immune serum or α -sAC antisera. Activity was measured by radioimmunoassay (Amersham) in the absence of any additions (striped bar), or in the presence of 40 mM NaHCO₃ or 100 μ M Forskolin. The results are shown in Fig 4b. This antisera specifically immunoprecipitated a bicarbonate stimulated AC activity from the cytosol of rat testis (Fig. 4b). The results also demonstrated that the immunoprecipitated activity was not forskolin responsive, and was therefore unlikely to be caused by cross-reacting tmACs in the immunoprecipitate. Data in Figure 4b are presented as pmol of cAMP formed over 20 minutes, and values represent averages of duplicate determinations with standard deviations indicated.

sAC activity is stimulated by bicarbonate. To examine the effect of bicarbonate on sAC catalytic activity, we constructed a stable HEK293 cell line expressing the full-length (sAC_{fl}) cDNA (HEK293/sAC_{fl}) and a cell line transfected with vector. The cell lines were starved for bicarbonate (grown in HEPES-buffered, NaHCO₃-free D-MEM under ambient CO₂) for 24 hours. Cellular cAMP accumulation and *in vitro* adenylyl cyclase activity in the presence of [³²P] ATP and MnCl₂ or MgCl₂ were assayed. *In vitro* adenylyl cyclase assays were performed at pH 7.5 in Tris-buffered solutions.

Addition of NaHCO₃ to the extracellular medium stimulated cAMP accumulation in HEK293/sAC_{fl} but not in vector transfected HEK293 cells (HEK293/V)(Fig. 5a). Bicarbonate increased cAMP production at the earliest time points tested, but accumulation was complete by 10 minutes. These data demonstrated that sAC can be activated by bicarbonate in the absence of any additional testis or sperm specific factors.

To delineate the regions of sAC that mediate bicarbonate activation, we constructed an additional stable cell line (HEK293/sAC_t) expressing a catalytically active, N-terminal truncation (sAC_t) consisting almost exclusively of the two catalytic domains which approximates the native 48 kDa sAC species. Bicarbonate also stimulated cAMP accumulation in HEK293/sAC_t revealing that bicarbonate stimulation of sAC activity did not require the large C terminal domain. Bicarbonate also activated heterologously expressed sAC *in vitro*. Adenylyl cyclase activity was stimulated in cellular lysates from HEK293/sAC_{fl} and from HEK293/sAC_t cells (Fig. 5b), further indicating that the enzyme is directly modulated by bicarbonate ions.

Bicarbonate activation of sAC is direct, specific, and pH independent.

Experiments were performed to demonstrate that bicarbonate acted directly on sAC and to exclude the possibility of accessory factors mediating activation in preparations using testis and stable cell lines. Purified recombinant sAC_t protein (sAC_t, amino acids 1-469, plus a C-terminal Hexahistidine tag was heterologously expressed in insect HiFive cells using the Bac-to-Bac Baculovirus Expression System (Life Technologies), and protein was purified by chromatography over Ni²⁺-NTA Sepharose Resin (Qiagen)) was assayed in the presence of 0-80 mM NaHCO₃ with 10 mM ATP and 40 mM MgCl₂. Purified enzyme was stimulated greater than seven-fold (Fig. 6a) with an EC₅₀ (25.4 ± 7.6 mM) within the physiologically relevant bicarbonate concentration in mammalian serum (22-26 mM) (Pitts, R.F., Physiology of the Kidney and Body Fluids (Year Book Medical Publishers, Inc., Chicago, ed., 3rd, 1974; Johnson, 1998). Kinetic parameters were determined using KinetAsyst II (IntelliKinetics). These results suggest that direct activation of sAC accounts for the observed intracellular increase in cAMP generation in sACexpressing cell lines (shown in Fig. 5a).

Purified sAC_t was also assayed as above at pHs 7-8.5 (final pH) (buffered by Tris-HCl) in the presence or absence of 40 mM NaHCO₃. The results shown in Figure 6b demonstrated that bicarbonate stimulation was not due to altered pH because both Mg²⁺-ATP alone and bicarbonate-stimulated sAC activities were completely insensitive to pH changes over the pH range 7.0-8.5.

Among mammalian adenylyl cyclases, sAC appears to be the only form regulated by bicarbonate ions. HEK293/V cells, which express endogenous tmACs, were unaffected by bicarbonate addition (see Fig. 5). Even when submaximally stimulated by forskolin, tmAC activity was insensitive to bicarbonate. Engineered soluble tmACV was constructed and purified as described (Scholich, K et al., Proc. Natl. Acad. Sci. USA, 1997, 94:2915) and assayed in the presence of MgCl₂ alone (basal), or with 50 mM NaHCO₃, or 100 μM Forskolin.

Data are presented in Figure 6c as pmol cAMP formed per minute per mg protein, and values represent triplicate determinations with standard deviations indicated. Results demonstrated that while a purified engineered soluble form of tmAC Type V was fully responsive to forskolin, it was completely insensitive to bicarbonate addition. Therefore, bicarbonate stimulation is not a general feature of all ACs, and mammalian cells possess two independently regulated cAMP signal transduction systems.

Activation of sAC by various salts. sAC activity was stimulated equally well by NaHCO_3 or KHCO_3 . The stimulatory effects of NaHCO_3 were successfully produced using bisulfite ion (Na_2SO_3 or NaHSO_3), which structurally resembles bicarbonate, but not with dissimilar ions, such as chloride (NaCl), sulfate (Na_2SO_4), or phosphate (Na_2HPO_4). The results are shown in Table 1.

Table 1
Activation of sAC by various salts.

| <u>Salt</u> ¹ | <u>% Basal</u> ² |
|---------------------------|-----------------------------|
| NaHSO_3 | 138±4.6 |
| Na_2SO_3 | 164±2.9 |
| NaCl | 93±11.8 |
| Na_2HPO_4 | 75±2.6 |
| Na_2SO_4 | 66±4.6 |
| NaHCO_3 | 411±5.3 |
| KHCO_3 | 412±6.1 |

¹Salts were used at 50 mM.

²Adenylyl cyclase activity shown relative to activity in the absence of any salts.

These data exclude Na^+ ion and simple alterations of ionic strength as regulators of sAC activity, and they indicate that bicarbonate, as opposed to CO_2 , directly binds to and activates sAC in a pH independent manner. However, because carbon dioxide is in equilibrium with bicarbonate, sAC and the cAMP signaling pathway may also indirectly monitor *in vivo* levels of carbon dioxide.

Bicarbonate activates cyanobacterial adenylyl cyclase. The two catalytic domains of sAC (C1 and C2) more closely resemble the active portions of cyanobacterial ACs than those from mammalian tmACs (Fig. 7a). cAMP is known to regulate respiration in cyanobacteria (Ohmori, K et al., Plant Cell Physiol., 1992, 33:21), but there is no known molecule which modulates their AC activity. In order to determine whether bicarbonate regulation of cAMP signaling is conserved in cyanobacteria, *Spirulina platensis* CyaC was expressed and purified as described (Kasahara, M. et al., Plant Cell Physiol., 1997, 38:828), and was assayed in the presence of 0-60 mM NaHCO_3 with 100 μM ATP and 5 mM MnCl_2 (Kasahara 1997; Kasahara and Ohmori, J. Biol. Chem., 1999, 274:15167). The AC activity of purified *Spirulina platensis* CyaC was stimulated 2.5 fold by the

presence of bicarbonate ions (Fig. 7b). Similar to mammalian sAC, bicarbonate stimulated cyanobacterial CyaC with an EC_{50} of 18.8 ± 1.6 mM and regulation was pH independent. These data demonstrated that cyanobacterial adenylyl cyclases were also bicarbonate sensitive.

5 **Example 3: sAC is an oncogene:**

Focus Formation Assay A full length cDNA clone from the library and the truncated clone as described above were expressed in mammalian cells under the control of the cytomegalovirus (CMV) promoter. The vector used, pBK-CMV is part of the λ ZAP library construction system (STRATAGENE). To generate a mammalian
10 expression vector from a library cDNA clone, we deleted a small piece of the vector DNA which removed a bacterial promoter sequence which brought the CMV promoter into proximity of the inserted cDNA.

For Rap1, we used wild type Rap1 in a CMV promoter vector (gift of Dr. Xin-Yun Huang, Department of Physiology, Weill Cornell Medical College) and for RAS,
15 we used v-Ras in CMV promoter vector (gift of Dr. Marvin Gershengorn, Department of Medicine, Weill Cornell Medical College). Vector control was pBK-CMV with no insert. The tmAC control was rat Type II adenylyl cyclase in pCIS expression plasmid and the Gs* control is the alpha subunit of Gs protein with a point mutation converting the glutamine at amino acid 227 to leucine expressed in pcDNA1.2 (gift of Drs. Henry Bourne
20 and Bruce Conklin, University of San Francisco).

One microgram of the indicated plasmids were transfected into low background (kind gift of Dr, Stuart Aaronson, Mt. Sinai Medical School) NIH 3T3 cells using lipofectamine (Life Technologies, Inc.) according to manufacturers instructions. Growth media (D-MEM plus 10% calf serum) was changed every three days and foci were
25 scored at the end of three weeks. For photographing, plates were stained with Giemsa stain as described previously (Zhou et al., 1994).

The truncated sAC_t transformed NIH 3T3 cells by focus-forming assay and produced as many foci as the oncogenic form of Ras. Furthermore, Rap1 protein, which selectively blocked transformation by oncogenic Ras proteins, also blocked transformation
30 by sAC_t suggesting sAC and Ras may share their biochemical mechanism of transformation. The results are shown in Table 3.

Table 3. Foci Formation in NIH3T3 cells

| Plasmid(s) (1µg) | Expt. #1 | Expt. #2 | Expt. #3* |
|------------------|----------|----------|-----------|
| Vector | 13 | 12 | 0 |
| v-Ras | 50 | 76 | 32 |
| sAC-t | 124 | 120 | 22 |
| sAC-fl | 26 | 47 | 0 |
| v-Ras+Rap1 | | | 2 |
| sAC+Rap1 | | | 1 |

Average number of Foci on at least 2 independent plates.

*Expt. 3 performed in Low Background NIH3T3 cells.

Full-length sAC was less oncogenic, inducing more foci than vector alone but less than sAC_t. By this criteria sAC is an oncogene.

Fig. 8 is a photograph of foci stained with Giemsa. The panels are NIH 3T3 cells transfected with the indicated plasmids. Foci appear as dark blotches of cells.

Soft Agar Assay We tested the ability of sAC_t and sAC_{fl} to support anchorage independent growth in soft agar. Low background NIH 3T3 cells were transfected as above. Two day, later, cells were trypsinized, counted and 10⁵ cells were replated into growth media plus 0.5% agarose in 60 mm tissue culture plates. Fresh media plus 0.5% agarose was overlaid each week and colony formation was scored after three weeks.

Table 4. Soft Agar Assay

| Plasmid | # of colonies |
|---------|---------------|
| Vector | 6 |
| v-Ras | 120 |
| sAC-t | 70 |
| sAC-fl | 50 |
| tmAC-2 | 9 |

Average of 2 plates

The results shown in Table 4 demonstrate that sAC is oncogenic; it confers the ability to grow in soft agar.

Expression of sAC in various cell lines and platelets Human embryonal kidney cells (HEK293) expressed full length sAC and its 120 kD and 48 kD isoforms.

5 Human lymphoblastoid T and B cells (Jurkat and 5/2, respectively) expressed predominantly the 48 kD isoform and human platelets predominantly the 45 kD and 48kD isoforms with no 120 kD isoform detectable. One mouse germ cell line (GC1) expressed all isoforms including 120 kD, a second mouse germ cell line (GC2) expressed all except the 120 kD isoform.

10 **sAC protein is elevated in human tumors** We compared sAC protein expression in human colon cancer samples and surrounding normal colon tissue. Western blot analysis is shown in Figure 9. Both α C1-C2 and α C-term antisera demonstrate that full-length sAC is upregulated in two out of three primary colon carcinomas tested. Additionally, α C1-C2 shows that the 120 kD isoform is also upregulated. Because less
15 stringent washing conditions were used in these Western blots, additional, nonspecific bands are seen, and we do not know yet whether the 45 kD and 48 kD isoforms of sAC are also upregulated in these human tumors.

Full length and the 120 kD isoform of sAC are upregulated in the colon of Min mice *Min* is an autosomal dominant mutation that predisposes mice to develop
20 intestinal tract adenomas. *Min* heterozygous (*Min*/+) mice develop, on average, more than 50 tumors throughout the entire length of their intestinal tract and rarely live past 150 days of age (Shoemaker, A.R. et al., BBA, 1997, 1332:F25-48). *Min* maps to a nonsense mutation in the adenomatous polyposis coli (APC) tumor suppressor gene. We compared sAC expression in colons from wild type littermates, 'normal' colon from *Min*/+ mice,
25 and tumors from *Min* mice. As with human colon carcinomas, both full-length sAC and the 120 kD isoform, but not the 48 kD isoform, are upregulated in tumors compared to wild type colon. Surprisingly, both sAC forms are also upregulated in *Min*/+ 'normal' colon which does not yet have visually detectable adenomas. We cannot exclude however that the *Min*/+ 'normal' colon tested contained microscopic adenomas. This 'normal'
30 colon still possesses a mutant APC gene thought to generate a dominant negative form of the protein, which contributes to a pre-cancerous state in these cells (Mahmoud, N.N. et al., Cancer Res., 1997, 57:5045-5050). Upregulation of sAC protein in these "pre-

cancerous" cells suggests sAC is a very early marker for tumorigenesis.

Example 4: sAC expression is inducible

The commercially available (Invitrogen) ecdysone inducible system (No, D. et al., PNAS, 1996, 93:3346-3351) is based on an insect hormone regulated transcriptional activator, the ecdysone receptor (EcR). This system takes advantage of the specificity of EcR for its own response element, and the lack of any cross-reactivity by any mammalian transcription factor. We stably introduced both the EcR and its requisite partner, Retinoid X Receptor (RXR) into NIH3T3 cells. Additionally, to confirm the efficacy of this expression system, we performed pilot experiments with 3T3 cells transiently transfected with both plasmids. Doubly transfected cells displayed a ponasterone A (a synthetic ecdysone analog) dependent increase in sAC activity. This induction was overcome by co-expression of sAC antisense (α sAA). The sequence was the entire sAC truncated (sAC trunc) sequence cloned in reverse orientation with respect to the promoter. The coding sequence for sAC trunc was approximately 1 through 1710 and the antisense was nucleotides 1710 through 1. For the antisense experiment, we transiently transfected three plasmids into NIH3T3 cells: 1) Ecdysone receptor/RXR expression plasmid; 2) sAC trunc expression plasmid (driven by ecdysone promoter) and 3) antisense expression plasmid (driven by ecdysone promoter). In the triply transfected cells, there was no increase in sAC activity following ponasterone A addition.

Example 5: Screening assay for sAC inhibitors

For this assay, MacConkey-maltose plates are prepared. To MacConkey agar base (Difco) add 40 mg MacConkey agar base per liter H₂O. Autoclave. Keep at 50°C. Make 20% maltose, and filter sterilize. Add 20% maltose per liter MacConkey agar base and the desired antibodies. Pour the plates.

For M63 plates: 1. Make 2X M63 basic salt: 27.2g KH₂PO₄, 4g(NH₄)₂SO₄ and 1 mg FeSO₄•7H₂O are added to 1 liter H₂O, adjust pH to 7.0 by KOH, autoclave to sterilize. Make 2X agar. 15g/500 ml Bacto-Agar and Autoclave. Make 1X M63-agar by adding 500 ml of 2X M63 basic salt and 2x agar after cooling at 50°C add 1 ml sterile 1 M MgSO₄, 25-30 ml 20% maltose, and 10 mg/ml arginine. Pour the plate.

Assay: Transform TP2000 (cya-) or WTC-3 (cya-, cpdA-) with a plasmid or the control plasmid. For negative control use: pProExHaH6. For positive control use

pProExHAH6-G_{st}Q-IC₁IIC₂. After transformation, grow up the cells overnight with antibiotics. Take 100 µl cells, spin down and wash once the cells with 1X M63 medium (essential, carry-over LB interferes the assay). Resuspend the cells with 1X M63 medium. Spot the M63-maltose or MacConkey-maltose plate which has 100 µM IPTG. The AC
5 complementation is best at 30°C, not 37°C.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the
10 foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

15 Various patents, patent applications, and publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED:

1 1. An isolated nucleic acid molecule encoding a soluble adenylyl cyclase,
2 wherein the nucleic acid molecule is selected from the group consisting of:

3 a) a nucleic acid which encodes a polypeptide having an amino acid
4 sequence as set out in SEQ ID NO: 1 or SEQ ID NO: 11, a splice variant thereof or an
5 allelic variant thereof;

6 b) a nucleic acid molecule which hybridizes under stringent conditions to a
7 nucleic acid having a sequence set out in SEQ ID NO: 2 or SEQ ID NO: 12;

8 c) a nucleic acid molecule which has at least a twenty nucleotide sequence
9 identical to a corresponding twenty nucleotide sequence as set out in SEQ ID NO:2 or SEQ
10 ID NO: 12; and

11 d) a nucleic acid sequence encod
12 ing a soluble polypeptide having an amino acid sequence sufficiently duplicative of the
13 soluble adenylyl cyclase encoded by SEQ ID NO: 1 or SEQ ID NO: 11 so that a
14 polypeptide expressed from the nucleic acid molecule is soluble in an isotonic buffered
15 nucleic solution free of surfactants, and has the biological property of catalyzing the
16 production of cyclic AMP, wherein the polypeptide expressed from the nucleic acid
17 molecule comprises a catalytic domain that is not more than 16% similar to a catalytic
18 domain of a mammalian transmembrane adenylyl cyclase as determined by CLUSTAL
19 sequence comparison.

1 2. An isolated nucleic acid molecule according to claim 1, which encodes a
2 mammalian soluble adenylyl cyclase.

1 3. An isolated nucleic acid molecule according to claim 2, which encodes a
2 human soluble adenylyl cyclase.

1 4. An isolated nucleic acid molecule according to claim 2, which encodes a
2 rat soluble adenylyl cyclase.

1 5. A vector comprising the nucleic acid molecule according to claim 1.

1 6. An expression vector comprising the nucleic acid molecule according to
2 claim 1 operably associated with an expression control sequence.

1 7. The vector of claim 6 which is a non-viral vector.

1 8. The vector of claim 7 wherein the vector is pBK-CMV.

1 9. A host cell comprising the expression vector according to claim 6.

1 10. The host cell according to claim 9, wherein the host cell is a
2 mammalian host cell.

1 11. The host cell according to claim 10, wherein the mammalian host cell
2 is a human host cell.

1 12. An oligonucleotide that specifically hybridizes under stringent
2 conditions to a nucleic acid having a sequence as set out in SEQ ID NO:1 or its
3 complement.

1 13. The oligonucleotide of claim 12 which has a sequence identical to at
2 least 20 continuous nucleotides of SEQ ID NO:2.

1 14. A method for producing a soluble adenylyl cyclase, which method
2 comprises isolating a soluble adenylyl cyclase expressed by the host cell of claim 9
3 cultured under conditions that permit expression of the soluble adenylyl cyclase by the host
4 cell.

1 15. The method of claim 14 wherein the soluble adenylyl cyclase is isolated
2 using an anti-soluble adenylyl cyclase specific antibody.

1 16. A method of modulating cell proliferation, which method comprises

2 administering a soluble adenylyl cyclase inhibitor compound to the cell in an amount
3 effective to inhibit or reduce unwanted cell proliferation.

1 17. A method of increasing insulin secretion of pancreatic islet cells, which
2 method comprises increasing soluble adenylyl cyclase activity.

1 18. A method of inducing capacitation of sperm comprising treating the
2 sperm with an agent that increases soluble adenylyl cyclase activity.

1 19. A method of inhibiting the fertilization of an ovum, which method
2 comprises inhibiting soluble adenylyl cyclase activity.

1 20. A method for reducing aqueous humor formation by administering to a
2 subject in need thereof a modulator of soluble adenylyl cyclase activity in an amount
3 effective to reduce or inhibit soluble adenylyl cyclase activity.

1 21. A method for quantifying bicarbonate in a body fluid sample, which
2 method comprises contacting the body fluid sample with soluble adenylyl cyclase and
3 detecting cAMP production, wherein the amount of cAMP produced is directly
4 correlatable to the amount of bicarbonate in the sample.

1 22. A method of screening for a modulator of sAC-induced signaling,
2 which method comprises detecting modulation of a signal of a sAC-induced signal
3 transduction pathway in a cell in the presence of a candidate compound wherein detection
4 of modulation of the signal indicates that the candidate compound is a modulator of sAC
5 induced signaling.

1 23. The method of claim 22, wherein the signal is cAMP generation.

1 24. A method of detecting the expression of soluble adenylyl cyclase in a
2 sample, which method comprises detecting the presence of a soluble adenylyl cyclase in a
3 sample contacted with a binding partner specific for soluble adenylyl cyclase wherein

4 detection of binding indicates that soluble adenylyl cyclase is present in the sample.

1 25. The method of claim 24, wherein the sample is a biological sample.

1 26. The method of claim 25, wherein the sample is tumor derived.

1 27. The method according to claim 24, wherein the sAC is sAC protein.

1 28. The method according to claim 27, wherein the binding partner specific
2 for sAC is an anti-sAC antibody.

1 29. The method according to claim 24, wherein the sAC is sAC mRNA.

1 30. The method according to claim 29, wherein the binding partner specific
2 for sAC is a sAC-specific oligonucleotide.

1 31. A method of diagnosing the likelihood or onset of, or for monitoring the
2 course and severity of a pathological condition associated with sAC activation, which
3 method comprises detecting an increase in the level of sAC in a biological sample from a
4 mammal suspected of suffering such a condition by the method of claim 23.

1 32. The method according to claim 31, wherein the condition is cancer.

1 33. A method of diagnosing the likelihood or onset of, or for monitoring
2 the course and severity of a pathological condition associated with a deficiency of sAC
3 activation, which method comprises detecting a decrease in the level of sAC in a biological
4 sample from a mammal suspected of suffering such a condition, by the method of claim
5 23.

1 34. The method according to claim 33, wherein the pathological condition
2 is diabetes.

1 35. The method according to claim 33, wherein the pathological condition
2 is infertility.

1 36. A method of treating a pathological condition associated with sAC
2 activation in a subject suffering from such a condition, which method comprises inhibiting
3 functional activation of sAC.

1 37. The method of claim 36, wherein inhibiting the functional activity
2 comprises administering an inhibitor of sAC.

1 38. The method of claim 36, wherein the pathological condition is
2 glaucoma.

1 39. A method of treating a pathological condition associated with an
2 increase in sAC activation in a subject suffering from such a condition, which method
3 comprises inhibiting functional activation of sAC.

1 40. The method of claim 39, wherein inhibiting the functional activity
2 comprises administering a specific sAC antisense molecule to cells that express functional
3 sAC.

1 41. The method of claim 39, wherein inhibiting the functional activity
2 comprises administering a protein inhibitor of sAC.

1 42. The method of claim 39, wherein the pathological condition is cancer.

1 43. A method of treating a pathological condition associated with a
2 deficiency of sAC activation in a subject suffering from such a condition, which method
3 comprises inducing functional activation of sAC.

1 44. The method according to claim 43, wherein sAC is activated by

2 expressing functional sAC from a vector delivered to the subject.

1 45. The method according to claim 43, wherein the pathological condition
2 is diabetes.

1 46. The method according to claim 43, wherein the pathological condition
2 is infertility.

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FIG. 1a

MSARRQELQDRAIVKIAHLPLDIVYGDESPERSVKCFDGVLFVDSIG
 FTATTEKFTAMTHDRGAELVZILNYIISAIVEKVLIFFGGDILKEAGDA 100
 LLALMKVERKQLKNIITVVIKCSLEIHGLFLAKEVEZEGLDIRVKIGLAAG
 HITMLVFGDETRNYFLVIGQAVDDVRLAQHMAQMDVILSPKCHQLCDRS 200
 MIEIERIPDQRAVKVSEFLKPPPTFNEDEFFAKCHAFMDYYPSSGDHKNFLR
 LACHLESDEPELESLOKYVMEIILKQIDDKQLRGYLSELRPVTIVFVNLM 300
 FKEQDKAEVIGSAIQAAACVHITSVLKVTRGQINKVFHEDKGCSELCVFGF
 PGEKAPDEITHALESAYDIEFDCSQVHKIRTVSIGVASGIVFCGIVGHTV 400
 RHEITVIGQKVHIAARMAYYPGIVTCDSTVDGNSNLPAYFFKZLPKKVM
 KGVADPGPVYQCLGLNEKVMFGMAYLICNRYEGYPLLGRVREIDYFMSTM 500
 KDFELMTNCSRVLMHYEGLPGYGKSQVLMEIEYLASQHENHRAVAIALTKIS
 FHQNFYTIQILMANVLGLDTCCKHYKERQTNLQNRVKTLLDDKYHCLLNDI 600
 FHVQFPVSRHSMRKIRKQKQLEALFHKILEQTVREERIIFIIDEAQFV
 DVASWAFIEKLIRSMPIFIVMSLCPFPETPCAAANAIMKNRNTTYITLGT 700
 HQPQEIIRDKVCVDLSVSSIPRELD SylVEGSCGIPYCEELLKNLDHHRIL 800
 LIFQQAEEAEKTNVTWNNLFKYSVKPTEDHYLYTSIAAGQKEACYLTSGV

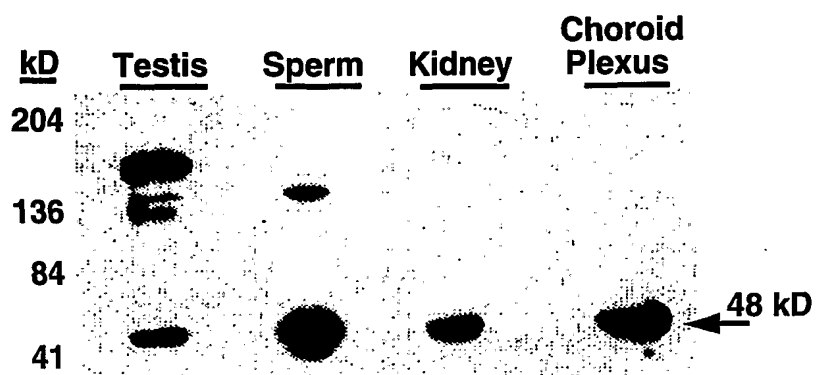
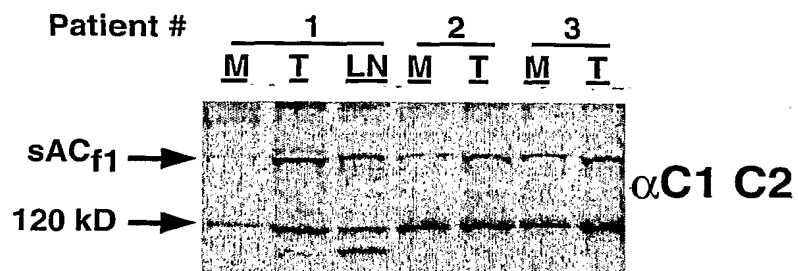
A ————— A

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FIG. 1b

| | | |
|---|---|------|
| A | RLKNLSPPASLKEISLVQLDSMSLSHQMLVRCAAIIGLTFTTELLFEILP | |
| | CWNMKMIKALATLVESNVFDCFRSSKDLQALKQNVTTFEVHYRSLSLK | 900 |
| | SKEGLAYSEEQREMEGEVIECRILRFCRPIMQKTAYELWLKDQKKVLH | |
| | LKCARFEESAHRCHCRNRDFIPYHHFIADIRLNTLDMDTVKKMVKSHG | 1000 |
| | FKTEDEVIFSKSEIPRKEKFPENISITETREKILHFFDNVVIKMRTSQDD | |
| | VIPLESCHCEELLQIVILPLAQHEVALEENNKALYYFLELASAYLILGDN | 1100 |
| | YNAYMYLGEGERLLKSLTNEDSWSQTFEYATFYSLKGEICFNMGMVLAK | |
| | KMLRKALKLLNRMFPCNLLSLTFQMHIEKNRSLSHFMNQHTQEGSLPGKKL | 1200 |
| | AQLFLQSSCFSLLWKIYSLNFFFHYKYGRLAAIMQMNTSLETQNNFQII | |
| | KAFLDPSLYRHLAGYEGVWFKEYEILVMEQLNLPLKGEAFEIMAYAADAL | 1300 |
| | GHIKFELTGHLDLAIELGSRRAHKMWSLLRNPKNKYHMLVLCRLSKPLFLKSR | |
| | KHLVQVLGWLNDLSVTEEHI FSKAFFYFVCLDIMLYSGFIYRTFEECLEF | 1400 |
| | IHHNEDNRILKFQSGLLGLYSCIAVWYARLQEWDNFYKFSNRAKTLVTR | |
| | RTPTVLYYEGISRYMEGQVLHLQKQIEEQAENAQDSGVELLKALETVAQ | 1500 |
| | NTTGPVFYPRLYHLMAYVCILMGDGHSCDFLNTALELSETQGNLLEKCH | |
| | LSMSKEWWSAPELTGQDQWLQTVLSLPSWDKIVSGNVTLODVQKNKFLMR | 1600 |
| | VNILDNPF | |

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FIG. 4a**FIG. 9**

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FIG. 2

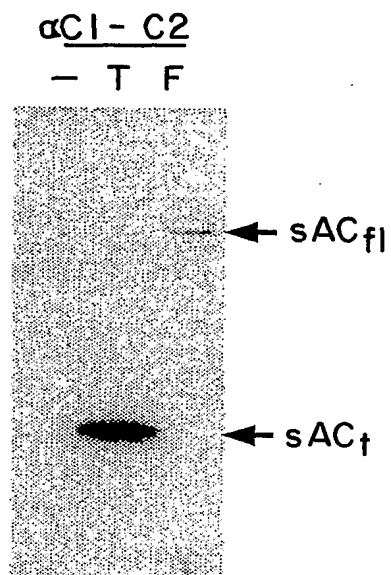
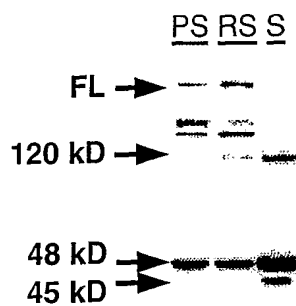


FIG. 3



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FIG. 4b

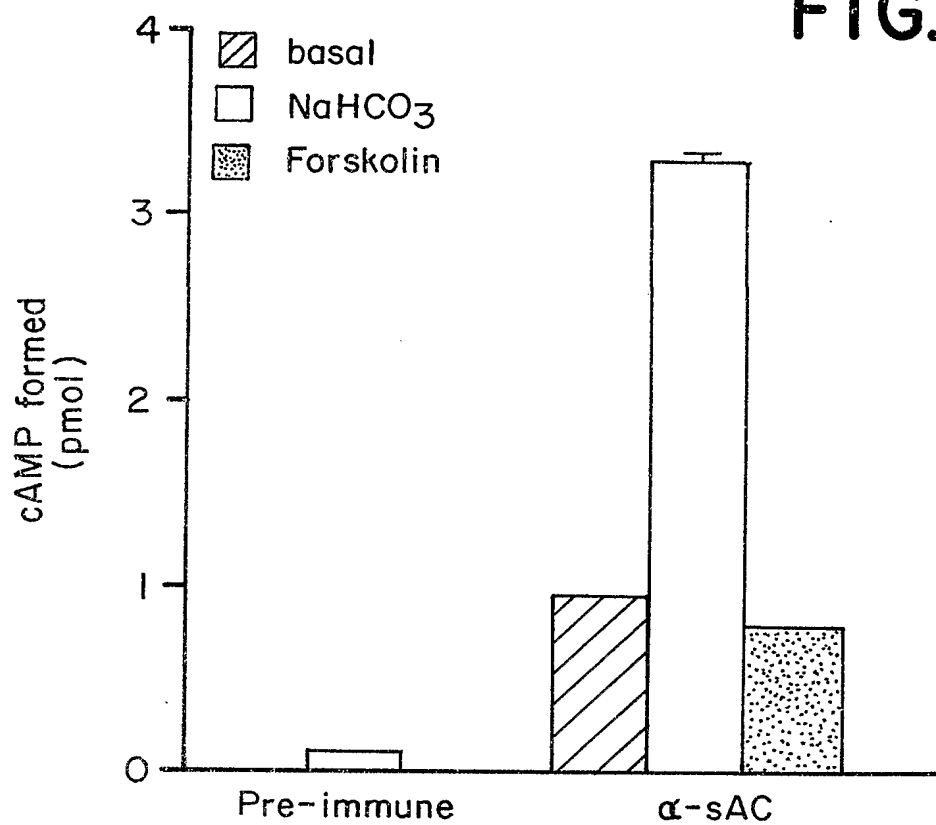
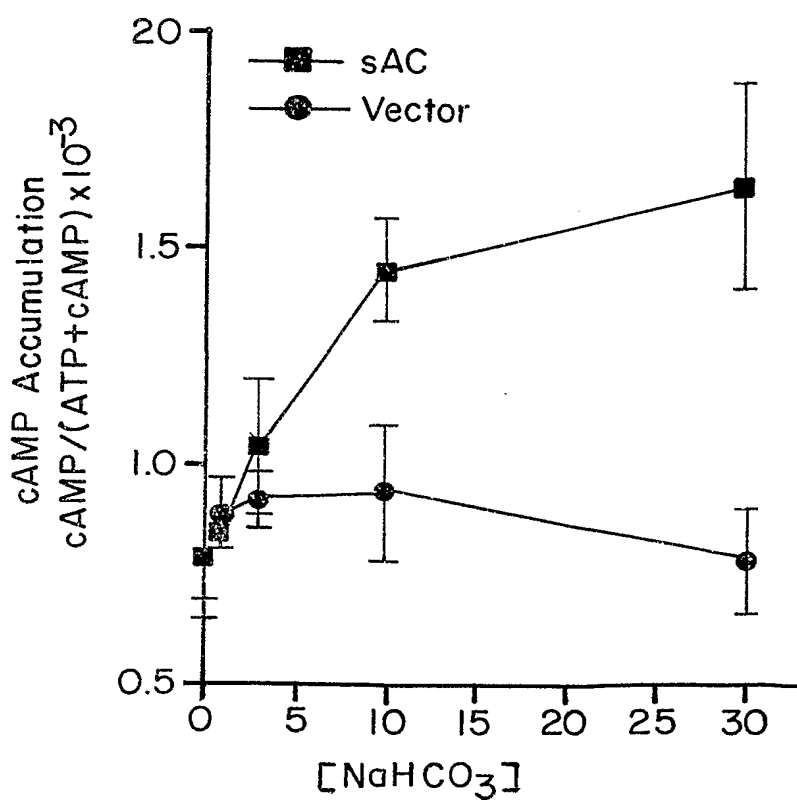


FIG. 5a



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FIG. 5b

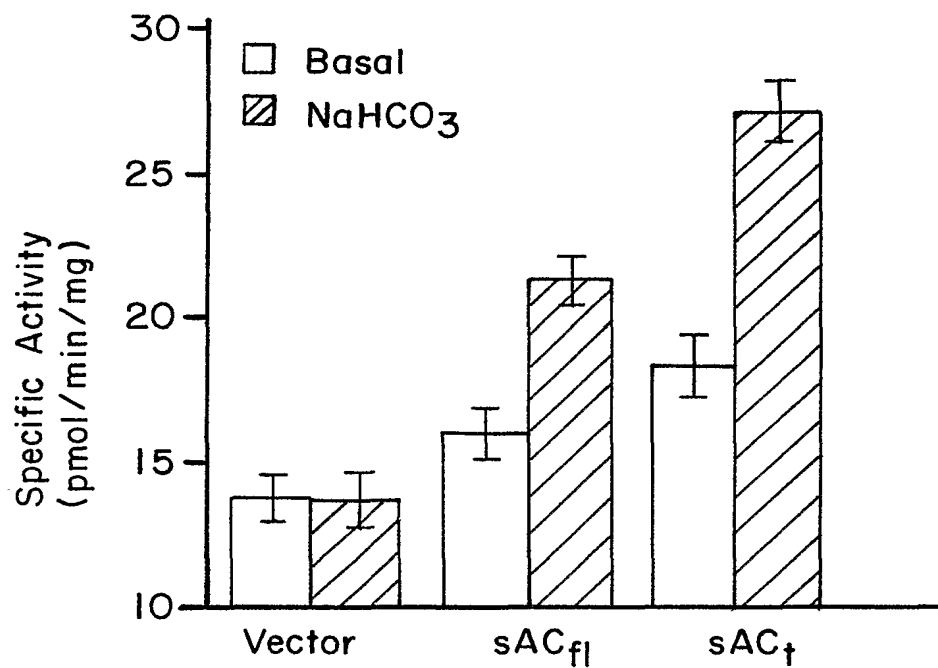
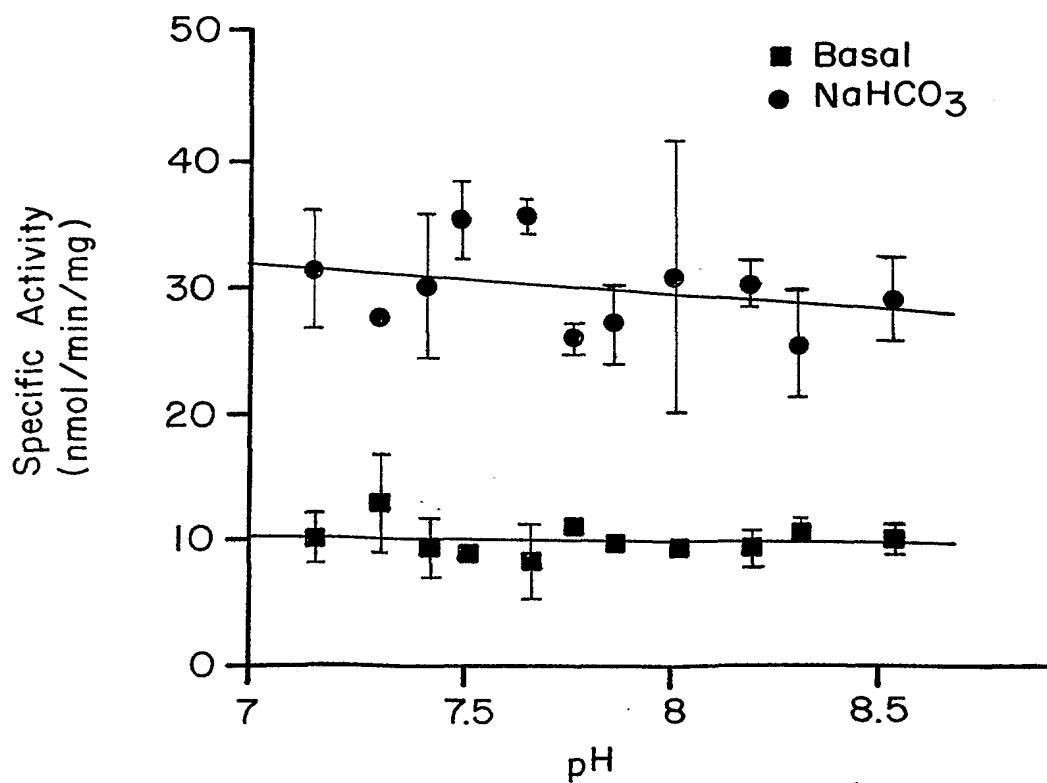
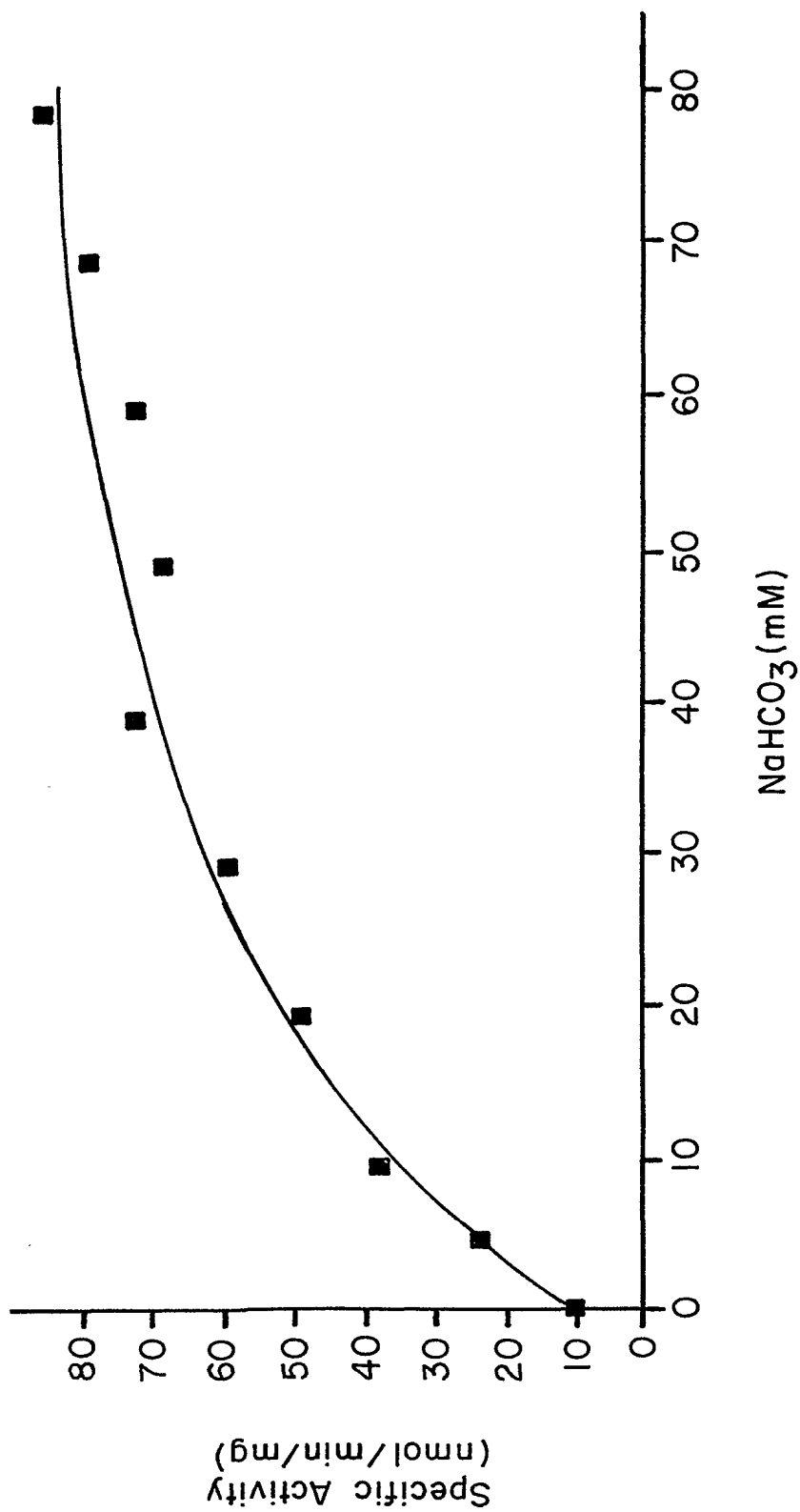


FIG. 6b

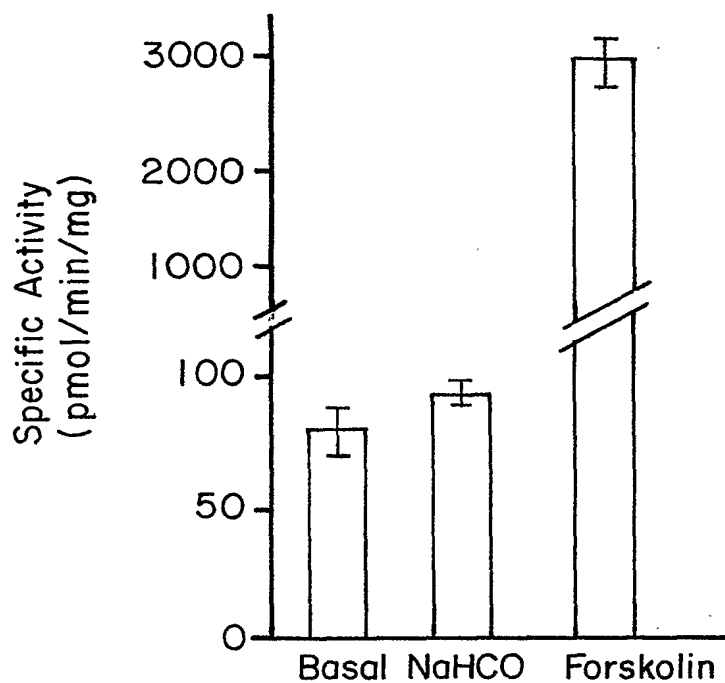
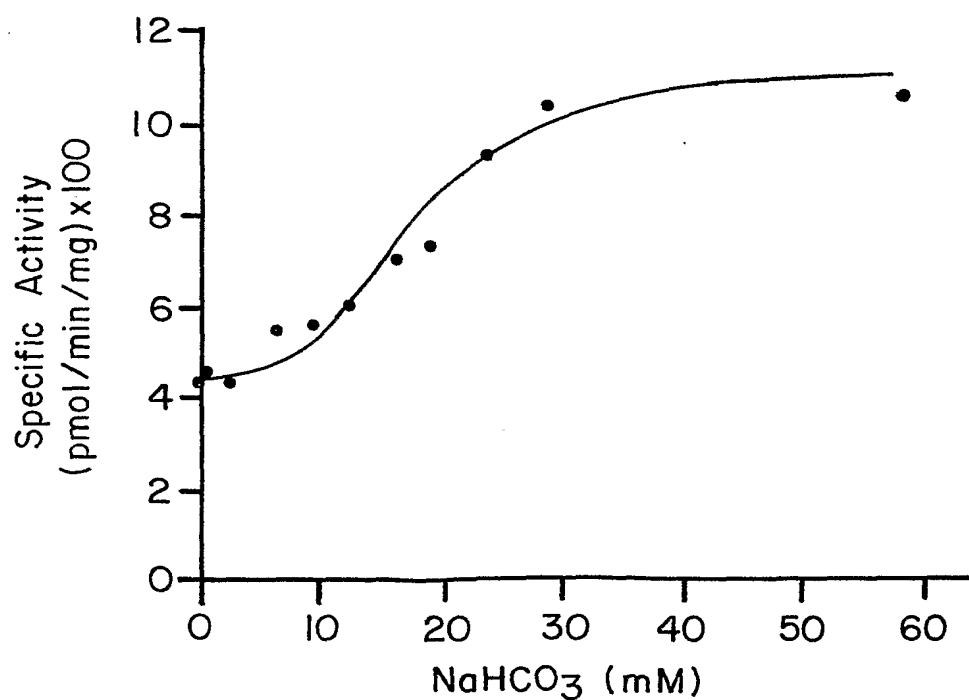


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FIG. 6a



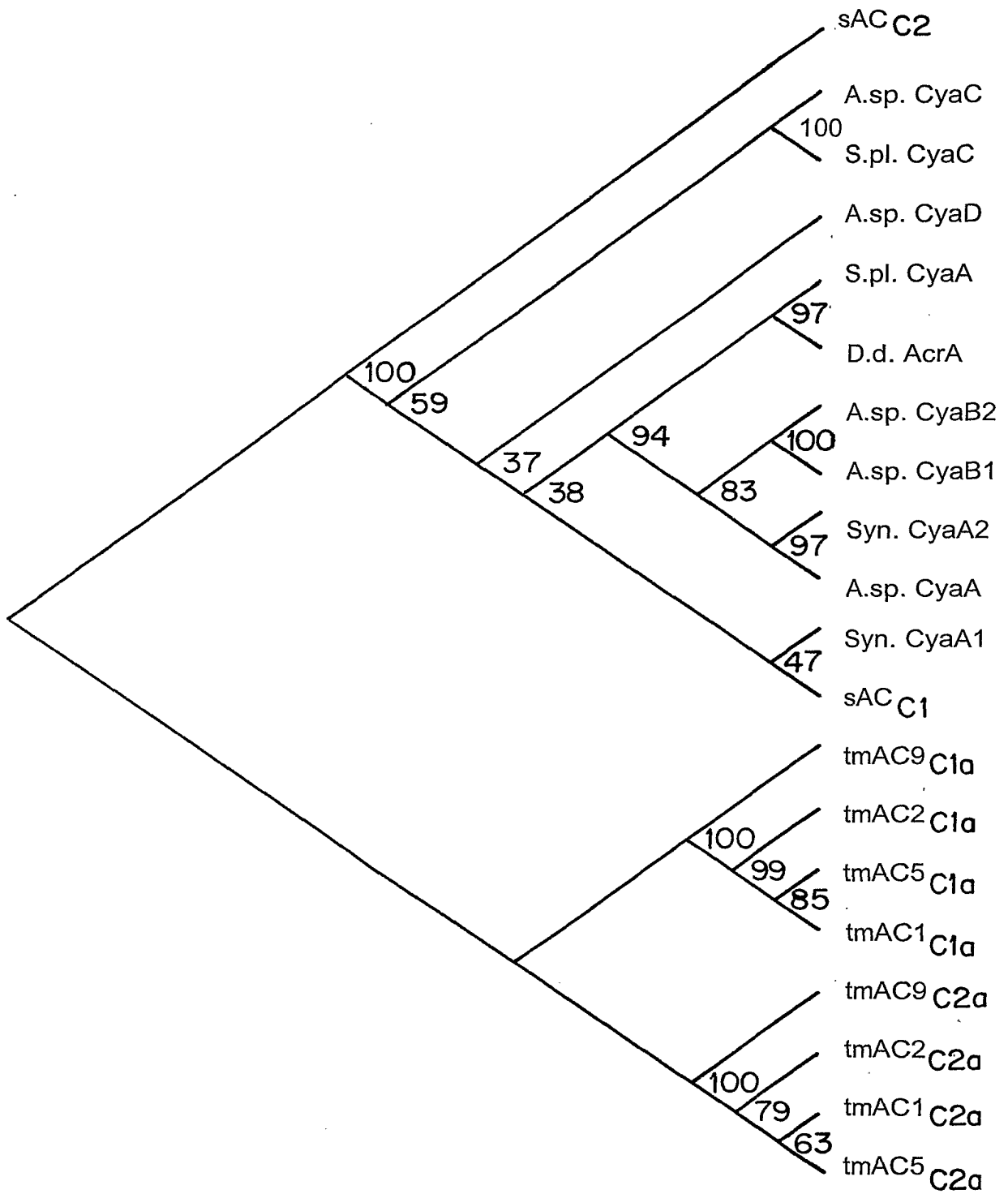
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FIG. 6c**FIG. 7b**

SUBSTITUTE SHEET (RULE 26)

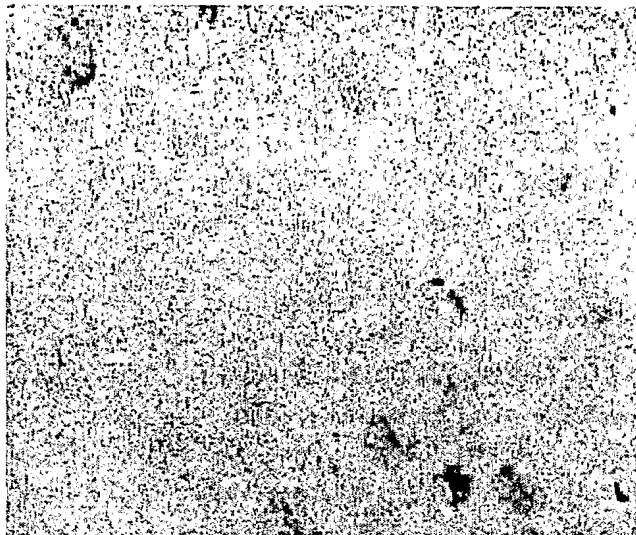
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FIG. 7a



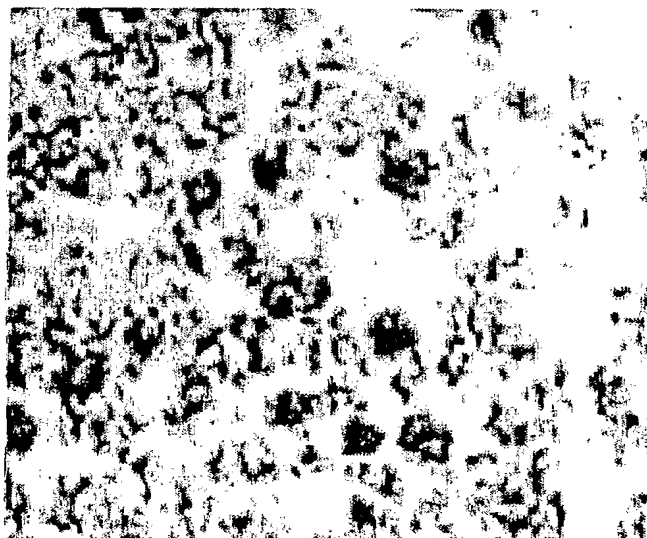
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FIG. 8a



Vector

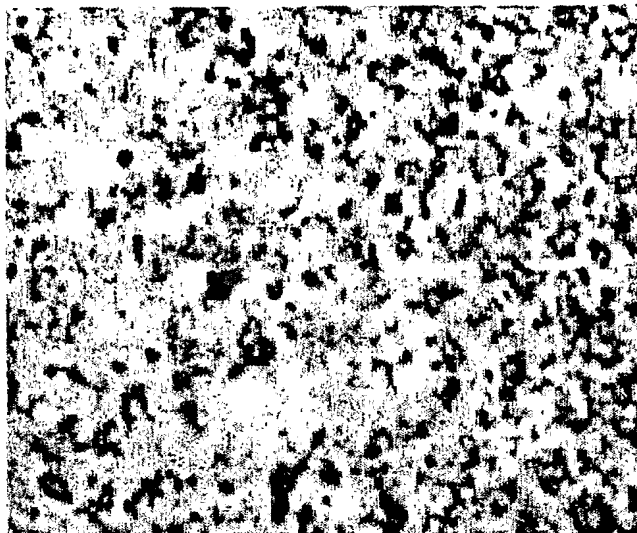
FIG. 8b



v-Ras

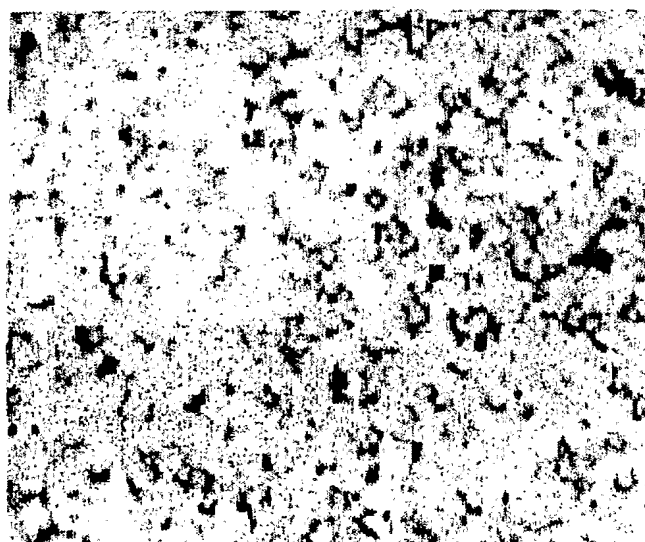
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FIG. 8c



sAC_↑

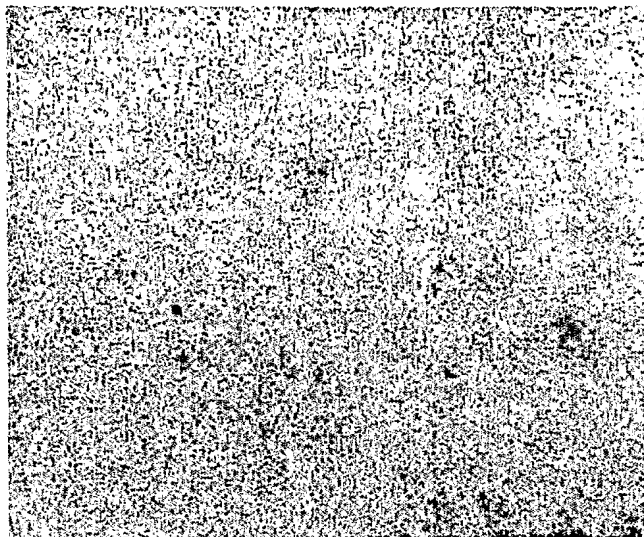
FIG. 8d



sAC_{fI}

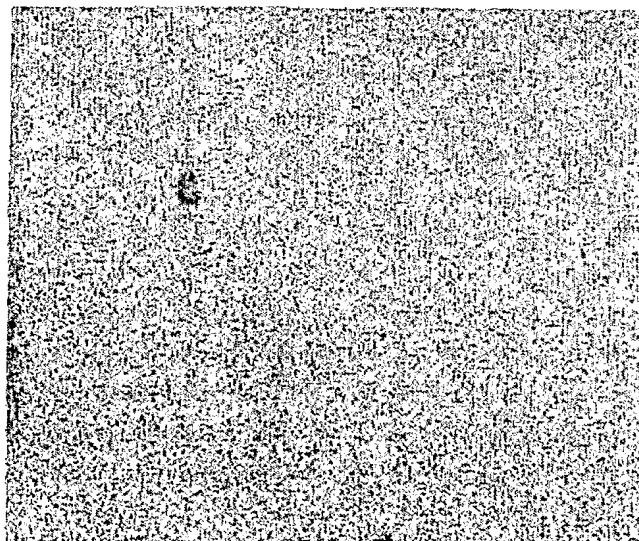
12/12

FIG. 8e



tmAC2

FIG. 8f



Gs α *

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Levin, Lonny R

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35 40 45

Ser Gly Phe Thr Ala Met Thr Glu Lys Phe Ser Thr Ala Met Tyr Met
50 55 60

Asp Arg Gly Ala Glu Gln Leu Val Glu Ile Leu Asn Tyr Tyr Ile Ser
65 70 75 80

Ala Ile Val Glu Lys Val Leu Ile Phe Gly Gly Asp Ile Leu Lys Phe

| | | | | | |
|---|---|-----|-----|-----|-----|
| | 85 | | 90 | | 95 |
| Ala Gly Asp | Ala Leu Leu Ala Leu Trp Lys Val Glu Arg Lys Gln Leu | | | | |
| | 100 | | 105 | | 110 |
| Lys Asn Ile Ile Thr Val Val Ile Lys Cys Ser Leu Glu Ile His Gly | | | | | |
| | 115 | | 120 | | 125 |
| Leu Phe Glu Ala Lys Glu Val Glu Glu Gly Leu Asp Ile Arg Val Lys | | | | | |
| | 130 | | 135 | | 140 |
| Ile Gly Leu Ala Ala Gly His Ile Thr Met Leu Val Phe Gly Asp Glu | | | | | |
| 145 | | 150 | | 155 | 160 |
| Thr Arg Asn Tyr Phe Leu Val Ile Gly Gln Ala Val Asp Asp Val Arg | | | | | |
| | 165 | | 170 | | 175 |
| Leu Ala Gln Asn Met Ala Gln Met Asn Asp Val Ile Leu Ser Pro Asn | | | | | |
| | 180 | | 185 | | 190 |
| Cys Trp Gln Leu Cys Asp Arg Ser Met Ile Glu Ile Glu Arg Ile Pro | | | | | |
| | 195 | | 200 | | 205 |
| Asp Gln Arg Ala Val Lys Val Ser Phe Leu Lys Pro Pro Pro Thr Phe | | | | | |
| | 210 | | 215 | | 220 |
| Asn Phe Asp Glu Phe Phe Ala Lys Cys Met Ala Phe Met Asp Tyr Tyr | | | | | |
| 225 | | 230 | | 235 | 240 |
| Pro Ser Gly Asp His Lys Asn Phe Leu Arg Leu Ala Cys Met Leu Glu | | | | | |
| | 245 | | 250 | | 255 |
| Ser Asp Pro Glu Leu Glu Leu Ser Leu Gln Lys Tyr Val Met Glu Ile | | | | | |
| | 260 | | 265 | | 270 |
| Ile Leu Lys Gln Ile Asp Asp Lys Gln Leu Arg Gly Tyr Leu Ser Glu | | | | | |
| | 275 | | 280 | | 285 |
| Leu Arg Pro Val Thr Ile Val Phe Val Asn Leu Met Phe Lys Glu Gln | | | | | |
| | 290 | | 295 | | 300 |
| Asp Lys Ala Glu Val Ile Gly Ser Ala Ile Gln Ala Ala Cys Val His | | | | | |
| 305 | | 310 | | 315 | 320 |
| Ile Thr Ser Val Leu Lys Val Phe Arg Gly Gln Ile Asn Lys Val Phe | | | | | |
| | 325 | | 330 | | 335 |
| Met Phe Asp Lys Gly Cys Ser Phe Leu Cys Val Phe Gly Phe Pro Gly | | | | | |

[illegible]

| | | |
|---|-----|---------|
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| 610 | 615 | 620 |
| Ala Leu Phe Met Lys Ile Leu Glu Gln Thr Val Arg Glu Glu Arg Ile | | |
| 625 | 630 | 635 640 |
| Ile Phe Ile Ile Asp Glu Ala Gln Phe Val Asp Val Ala Ser Trp Ala | | |
| | 645 | 650 655 |
| Phe Ile Glu Lys Leu Ile Arg Ser Met Pro Ile Phe Ile Val Met Ser | | |
| | 660 | 665 670 |
| Leu Cys Pro Phe Pro Glu Thr Pro Cys Ala Ala Ala Asn Ala Ile Met | | |
| | 675 | 680 685 |
| Lys Asn Arg Asn Thr Thr Tyr Ile Thr Leu Gly Thr Met Gln Pro Gln | | |
| | 690 | 695 700 |
| Glu Ile Arg Asp Lys Val Cys Val Asp Leu Ser Val Ser Ser Ile Pro | | |
| 705 | 710 | 715 720 |
| Arg Glu Leu Asp Ser Tyr Leu Val Glu Gly Ser Cys Gly Ile Pro Tyr | | |
| | 725 | 730 735 |
| Tyr Cys Glu Glu Leu Leu Lys Asn Leu Asp His His Arg Ile Leu Ile | | |
| | 740 | 745 750 |
| Phe Gln Gln Ala Glu Ala Glu Glu Lys Thr Asn Val Thr Trp Asn Asn | | |
| | 755 | 760 765 |
| Leu Phe Lys Tyr Ser Val Lys Pro Thr Glu Asp Met Tyr Leu Tyr Thr | | |
| | 770 | 775 780 |
| Ser Ile Ala Ala Gly Gln Lys Glu Ala Cys Tyr Leu Thr Ser Gly Val | | |
| 785 | 790 | 795 800 |
| Arg Leu Lys Asn Leu Ser Pro Pro Ala Ser Leu Lys Glu Ile Ser Leu | | |
| | 805 | 810 815 |
| Val Gln Leu Asp Ser Met Ser Leu Ser His Gln Met Leu Val Arg Cys | | |
| | 820 | 825 830 |
| Ala Ala Ile Ile Gly Leu Thr Phe Thr Thr Glu Leu Leu Phe Glu Ile | | |
| | 835 | 840 845 |
| Leu Pro Cys Trp Asn Met Lys Met Met Ile Lys Ala Leu Ala Thr Leu | | |

| | | | | |
|---|--|------|--|------|
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| Val Glu Ser Asn Val Phe Asp Cys Phe Arg Ser Ser Lys Asp Leu Gln | | | | |
| 865 | | 870 | | 875 |
| | | | | 880 |
| Leu Ala Leu Lys Gln Asn Val Thr Thr Phe Glu Val His Tyr Arg Ser | | | | |
| | | 885 | | 890 |
| | | | | 895 |
| Leu Ser Leu Lys Ser Lys Glu Gly Leu Ala Tyr Ser Glu Glu Glu Gln | | | | |
| | | 900 | | 905 |
| | | | | 910 |
| Leu Arg Glu Met Glu Gly Glu Val Ile Glu Cys Arg Ile Leu Arg Phe | | | | |
| | | 915 | | 920 |
| | | | | 925 |
| Cys Arg Pro Ile Met Gln Lys Thr Ala Tyr Glu Leu Trp Leu Lys Asp | | | | |
| | | 930 | | 935 |
| | | | | 940 |
| Gln Lys Lys Val Leu His Leu Lys Cys Ala Arg Phe Leu Glu Glu Ser | | | | |
| | | 945 | | 950 |
| | | | | 955 |
| | | | | 960 |
| Ala His Arg Cys Asn His Cys Arg Asn Arg Asp Phe Ile Pro Tyr His | | | | |
| | | 965 | | 970 |
| | | | | 975 |
| His Phe Ile Ala Asp Ile Arg Leu Asn Thr Leu Asp Met Asp Thr Val | | | | |
| | | 980 | | 985 |
| | | | | 990 |
| Lys Lys Met Val Lys Ser His Gly Phe Lys Thr Glu Asp Glu Val Ile | | | | |
| | | 995 | | 1000 |
| | | | | 1005 |
| Phe Ser Lys Ser Glu Ile Pro Arg Lys Phe Lys Phe Pro Glu Asn Ile | | | | |
| | | 1010 | | 1015 |
| | | | | 1020 |
| Ser Ile Thr Glu Thr Arg Glu Lys Ile Leu His Phe Phe Asp Asn Val | | | | |
| | | 1025 | | 1030 |
| | | | | 1035 |
| | | | | 1040 |
| Ile Ile Lys Met Arg Thr Ser Gln Asp Asp Val Ile Pro Leu Glu Ser | | | | |
| | | 1045 | | 1050 |
| | | | | 1055 |
| Cys His Cys Glu Glu Leu Leu Gln Ile Val Ile Leu Pro Leu Ala Gln | | | | |
| | | 1060 | | 1065 |
| | | | | 1070 |
| His Phe Val Ala Leu Glu Glu Asn Asn Lys Ala Leu Tyr Tyr Phe Leu | | | | |
| | | 1075 | | 1080 |
| | | | | 1085 |
| Glu Leu Ala Ser Ala Tyr Leu Ile Leu Gly Asp Asn Tyr Asn Ala Tyr | | | | |
| | | 1090 | | 1095 |
| | | | | 1100 |
| Met Tyr Leu Gly Glu Gly Glu Arg Leu Leu Lys Ser Leu Thr Asn Glu | | | | |

| | | | |
|---|------|------|------|
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| Gly Glu Ile Cys Phe Asn Met Gly Gln Met Val Leu Ala Lys Lys Met | 1140 | 1145 | 1150 |
| Leu Arg Lys Ala Leu Lys Leu Leu Asn Arg Met Phe Pro Cys Asn Leu | 1155 | 1160 | 1165 |
| Leu Ser Leu Thr Phe Gln Met His Ile Glu Lys Asn Arg Leu Ser His | 1170 | 1175 | 1180 |
| Phe Met Asn Gln His Thr Gln Glu Gly Ser Leu Pro Gly Lys Lys Leu | 1185 | 1190 | 1195 |
| Ala Gln Leu Phe Leu Gln Ser Ser Cys Phe Ser Leu Leu Trp Lys Ile | 1205 | 1210 | 1215 |
| Tyr Ser Leu Asn Phe Phe Phe His Tyr Lys Tyr Tyr Gly Arg Leu Ala | 1220 | 1225 | 1230 |
| Ala Ile Met Gln Met Asn Thr Ser Leu Glu Thr Gln Asn Asn Phe Gln | 1235 | 1240 | 1245 |
| Ile Ile Lys Ala Phe Leu Asp Phe Ser Leu Tyr Arg His Leu Ala Gly | 1250 | 1255 | 1260 |
| Tyr Glu Gly Val Trp Phe Lys Tyr Glu Ile Leu Val Met Glu Gln Leu | 1265 | 1270 | 1275 |
| Leu Asn Leu Pro Leu Lys Gly Glu Ala Phe Glu Ile Met Ala Tyr Ala | 1285 | 1290 | 1295 |
| Ala Asp Ala Leu Gly His Ile Lys Phe Leu Thr Gly His Leu Asp Leu | 1300 | 1305 | 1310 |
| Ala Ile Glu Leu Gly Ser Arg Ala His Lys Met Trp Ser Leu Leu Arg | 1315 | 1320 | 1325 |
| Asn Pro Asn Lys Tyr His Met Val Leu Cys Arg Leu Ser Lys Pro Leu | 1330 | 1335 | 1340 |
| Phe Leu Lys Ser Arg Tyr Lys His Leu Val Gln Val Leu Gly Trp Leu | 1345 | 1350 | 1355 |
| Trp Asp Leu Ser Val Thr Glu Glu His Ile Phe Ser Lys Ala Phe Phe | | | |

| | | |
|---|------|------|
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| Tyr Phe Val Cys Leu Asp Ile Met Leu Tyr Ser Gly Phe Ile Tyr Arg | | |
| 1380 | 1385 | 1390 |
| Thr Phe Glu Glu Cys Leu Glu Phe Ile His His Asn Glu Asp Asn Arg | | |
| 1395 | 1400 | 1405 |
| Ile Leu Lys Phe Gln Ser Gly Leu Leu Leu Gly Leu Tyr Ser Cys Ile | | |
| 1410 | 1415 | 1420 |
| Ala Val Trp Tyr Ala Arg Leu Gln Glu Trp Asp Asn Phe Tyr Lys Phe | | |
| 1425 | 1430 | 1435 |
| Ser Asn Arg Ala Lys Thr Leu Val Thr Arg Arg Thr Pro Thr Val Leu | | |
| 1445 | 1450 | 1455 |
| Tyr Tyr Glu Gly Ile Ser Arg Tyr Met Glu Gly Gln Val Leu His Leu | | |
| 1460 | 1465 | 1470 |
| Gln Lys Gln Ile Glu Glu Gln Ala Glu Asn Ala Gln Asp Ser Gly Val | | |
| 1475 | 1480 | 1485 |
| Glu Leu Leu Lys Ala Leu Glu Thr Leu Val Ala Gln Asn Thr Thr Gly | | |
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| Pro Val Phe Tyr Pro Arg Leu Tyr His Leu Met Ala Tyr Val Cys Ile | | |
| 1505 | 1510 | 1515 |
| Leu Met Gly Asp Gly His Ser Cys Asp Phe Phe Leu Asn Thr Ala Leu | | |
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| 1540 | 1545 | 1550 |
| Met Ser Lys Glu Trp Trp Tyr Ser Ala Pro Glu Leu Thr Gly Asp Gln | | |
| 1555 | 1560 | 1565 |
| Trp Leu Gln Thr Val Leu Ser Leu Pro Ser Trp Asp Lys Ile Val Ser | | |
| 1570 | 1575 | 1580 |
| Gly Asn Val Thr Leu Gln Asp Val Gln Lys Asn Lys Phe Leu Met Arg | | |
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<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:tryptic peptide

<400> 3

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5

<210> 4

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:tryptic peptide

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Glu Tyr Thr Val Ile Gly Gln Lys

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<210> 5

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:tryptic peptide

<220>

<221> VARIANT

<222> (5)

<223> fully degenerate for either leucine or glutamate

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Met Glu Leu Glu Xaa Asp Pro Glu

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<210> 6

<211> 20
<212> DNA
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<220>
<223> Description of Artificial Sequence:primer

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<210> 7
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

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<210> 8
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<212> DNA
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<223> Description of Artificial Sequence:primer

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cctgcttctc cctgctgtg 19

<210> 9
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<212> DNA
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<220>
<223> Description of Artificial Sequence:primer

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<210> 10

<211> 14

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence:N-terminal
region soluble adenylyl cyclase

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<210> 11

<211> 1610

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human soluble
adenylyl cyclase

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1

5

10

15

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20

25

30

Arg Pro Phe Met Asp Tyr Phe Asp Gly Val Leu Met Phe Val Asp Ile

35

40

45

Ser Gly Phe Thr Ala Met Thr Glu Lys Phe Ser Ser Ala Met Tyr Met

50

55

60

Asp Arg Gly Ala Glu Gln Leu Val Glu Ile Leu Asn Tyr His Ile Ser

65

70

75

80

Ala Ile Val Glu Lys Val Leu Ile Phe Gly Gly Asp Ile Leu Lys Phe

85

90

95

Ala Gly Asp Ala Leu Leu Ala Leu Trp Arg Val Glu Arg Lys Gln Leu

100

105

110

Lys Asn Ile Ile Thr Val Val Ile Lys Cys Ser Leu Glu Ile His Gly

115

120

125

Leu Phe Glu Thr Gln Glu Trp Glu Glu Gly Leu Asp Ile Arg Val Lys

| | | | |
|---|-----|-----|-----|
| Ile Gly Leu Ala Ala Gly His Ile Ser Met Leu Val Phe Gly Asp Glu | 130 | 135 | 140 |
| 145 | | 150 | 155 |
| Thr His Ser His Phe Leu Val Ile Gly Gln Ala Val Asp Asp Val Arg | | | |
| | 165 | | 170 |
| Leu Ala Gln Asn Met Ala Gln Met Asn Asp Val Ile Leu Ser Pro Asn | | | |
| | 180 | 185 | 190 |
| Cys Trp Gln Leu Cys Asp Arg Ser Met Ile Glu Ile Glu Ser Val Pro | | | |
| | 195 | 200 | 205 |
| Asp Gln Arg Ala Val Lys Val Asn Phe Leu Lys Pro Pro Pro Asn Phe | | | |
| | 210 | 215 | 220 |
| Asn Phe Asp Glu Phe Phe Thr Lys Cys Thr Thr Phe Met His Tyr Tyr | | | |
| 225 | | 230 | 235 |
| Pro Ser Gly Glu His Lys Asn Leu Leu Arg Leu Ala Cys Thr Leu Lys | | | |
| | 245 | | 250 |
| Pro Asp Pro Glu Leu Glu Met Ser Leu Gln Lys Tyr Val Met Glu Ser | | | |
| | 260 | 265 | 270 |
| Ile Leu Lys Gln Ile Asp Asn Lys Gln Leu Gln Gly Tyr Leu Ser Glu | | | |
| | 275 | 280 | 285 |
| Leu Arg Pro Val Thr Ile Val Phe Val Asn Leu Met Phe Glu Asp Gln | | | |
| | 290 | 295 | 300 |
| Asp Lys Ala Glu Glu Ile Gly Pro Ala Ile Gln Asp Ala Tyr Met His | | | |
| 305 | | 310 | 315 |
| Ile Thr Ser Val Leu Lys Ile Phe Gln Gly Gln Ile Asn Lys Val Phe | | | |
| | 325 | | 330 |
| Met Phe Asp Lys Gly Cys Ser Phe Leu Cys Val Phe Gly Phe Pro Gly | | | |
| | 340 | 345 | 350 |
| Glu Lys Val Pro Asp Glu Leu Thr His Ala Leu Glu Cys Ala Met Asp | | | |
| | 355 | 360 | 365 |
| Ile Phe Asp Phe Cys Ser Gln Val His Lys Ile Gln Thr Val Ser Ile | | | |
| | 370 | 375 | 380 |
| Gly Val Ala Ser Gly Ile Val Phe Cys Gly Ile Val Gly His Thr Val | | | |

| | | | | | | |
|---|-----|-----|-----|-----|-----|-----|
| 385 | | 390 | | 395 | | 400 |
| Arg His Glu Tyr Thr Val Ile Gly Gln Lys Val Asn Leu Ala Ala Arg | | | | | | |
| | 405 | | 410 | | 415 | |
| Met Met Met Tyr Tyr Pro Gly Ile Val Thr Cys Asp Ser Val Thr Tyr | | | | | | |
| | 420 | | 425 | | 430 | |
| Asn Gly Ser Asn Leu Pro Ala Tyr Phe Phe Lys Glu Leu Pro Lys Lys | | | | | | |
| | 435 | | 440 | | 445 | |
| Val Met Lys Gly Val Ala Asp Ser Gly Pro Leu Tyr Gln Tyr Trp Gly | | | | | | |
| | 450 | | 455 | | 460 | |
| Arg Thr Glu Lys Val Met Phe Gly Met Ala Cys Leu Ile Cys Asn Arg | | | | | | |
| 465 | | 470 | | 475 | | 480 |
| Lys Glu Asp Tyr Pro Leu Leu Gly Arg Asn Lys Glu Ile Asn Tyr Phe | | | | | | |
| | 485 | | 490 | | 495 | |
| Met Tyr Thr Met Lys Lys Phe Leu Ile Ser Asn Ser Ser Gln Val Leu | | | | | | |
| | 500 | | 505 | | 510 | |
| Met Tyr Glu Gly Leu Pro Gly Tyr Gly Lys Ser Gln Ile Leu Met Lys | | | | | | |
| | 515 | | 520 | | 525 | |
| Ile Glu Tyr Leu Ala Gln Gly Lys Asn His Arg Ile Ile Ala Ile Ser | | | | | | |
| | 530 | | 535 | | 540 | |
| Leu Asn Lys Ile Ser Phe His Gln Thr Phe Tyr Thr Ile Gln Met Phe | | | | | | |
| 545 | | 550 | | 555 | | 560 |
| Met Ala Asn Val Leu Gly Leu Asp Thr Cys Lys His Tyr Lys Glu Arg | | | | | | |
| | 565 | | 570 | | 575 | |
| Gln Thr Asn Leu Arg Asn Lys Val Met Thr Leu Leu Asp Glu Lys Phe | | | | | | |
| | 580 | | 585 | | 590 | |
| Tyr Cys Leu Leu Asn Asp Ile Phe His Val Gln Phe Pro Ile Ser Arg | | | | | | |
| | 595 | | 600 | | 605 | |
| Glu Ile Ser Arg Met Ser Thr Leu Lys Lys Gln Lys Gln Leu Glu Ile | | | | | | |
| | 610 | | 615 | | 620 | |
| Leu Phe Met Lys Ile Leu Lys Leu Ile Val Lys Glu Glu Arg Ile Ile | | | | | | |
| 625 | | 630 | | 635 | | 640 |
| Phe Ile Ile Asp Glu Ala Gln Phe Val Asp Ser Thr Ser Trp Arg Phe | | | | | | |

| | 645 | | | | | | | 650 | | | | | 655 | | | |
|---|-----|--|--|--|--|--|--|-----|--|--|--|--|-----|--|--|--|
| Met Glu Lys Leu Ile Arg Thr Leu Pro Ile Phe Ile Ile Met Ser Leu | | | | | | | | | | | | | | | | |
| | 660 | | | | | | | 665 | | | | | 670 | | | |
| Cys Pro Phe Val Asn Ile Pro Cys Ala Ala Ala Arg Ala Val Ile Lys | | | | | | | | | | | | | | | | |
| | 675 | | | | | | | 680 | | | | | 685 | | | |
| Asn Arg Asn Thr Thr Tyr Ile Val Val Gly Ala Val Gln Pro Asn Asp | | | | | | | | | | | | | | | | |
| | 690 | | | | | | | 695 | | | | | 700 | | | |
| Ile Ser Asn Lys Ile Cys Leu Asp Leu Asn Val Ser Cys Ile Ser Lys | | | | | | | | | | | | | | | | |
| | 705 | | | | | | | 710 | | | | | 715 | | | |
| Glu Leu Asp Ser Tyr Leu Gly Glu Gly Ser Cys Gly Ile Pro Phe Tyr | | | | | | | | | | | | | | | | |
| | 725 | | | | | | | 730 | | | | | 735 | | | |
| Cys Glu Glu Leu Leu Lys Asn Leu Glu His His Glu Val Leu Val Phe | | | | | | | | | | | | | | | | |
| | 740 | | | | | | | 745 | | | | | 750 | | | |
| Gln Gln Thr Glu Ser Glu Glu Lys Thr Asn Arg Thr Trp Asn Asn Leu | | | | | | | | | | | | | | | | |
| | 755 | | | | | | | 760 | | | | | 765 | | | |
| Phe Lys Tyr Ser Ile Lys Leu Thr Glu Lys Leu Asn Met Val Thr Leu | | | | | | | | | | | | | | | | |
| | 770 | | | | | | | 775 | | | | | 780 | | | |
| His Ser Asp Lys Glu Ser Glu Glu Val Cys His Leu Thr Ser Gly Val | | | | | | | | | | | | | | | | |
| | 785 | | | | | | | 790 | | | | | 795 | | | |
| Arg Leu Lys Asn Leu Ser Pro Pro Thr Ser Leu Lys Glu Ile Ser Leu | | | | | | | | | | | | | | | | |
| | 805 | | | | | | | 810 | | | | | 815 | | | |
| Ile Gln Leu Asp Ser Met Arg Leu Ser His Gln Met Leu Val Arg Cys | | | | | | | | | | | | | | | | |
| | 820 | | | | | | | 825 | | | | | 830 | | | |
| Ala Ala Ile Ile Gly Leu Thr Phe Thr Thr Glu Leu Leu Phe Glu Ile | | | | | | | | | | | | | | | | |
| | 835 | | | | | | | 840 | | | | | 845 | | | |
| Leu Pro Cys Trp Asn Met Lys Met Met Ile Lys Thr Leu Ala Thr Leu | | | | | | | | | | | | | | | | |
| | 850 | | | | | | | 855 | | | | | 860 | | | |
| Val Glu Ser Asn Ile Phe Tyr Cys Phe Arg Asn Gly Lys Glu Leu Gln | | | | | | | | | | | | | | | | |
| | 865 | | | | | | | 870 | | | | | 875 | | | |
| Lys Ala Leu Lys Gln Asn Asp Pro Ser Phe Glu Val His Tyr Arg Ser | | | | | | | | | | | | | | | | |
| | 885 | | | | | | | 890 | | | | | 895 | | | |
| Leu Ser Leu Lys Pro Ser Glu Gly Met Asp His Gly Glu Glu Glu Gln | | | | | | | | | | | | | | | | |

| | | |
|---|------|------|
| 900 | 905 | 910 |
| Leu Arg Glu Leu Glu Asn Glu Val Ile Glu Cys His Arg Ile Arg Phe | | |
| 915 | 920 | 925 |
| Cys Asn Pro Met Met Gln Lys Thr Ala Tyr Glu Leu Trp Leu Lys Asp | | |
| 930 | 935 | 940 |
| Gln Arg Lys Ala Met His Leu Lys Cys Ala Arg Phe Leu Glu Glu Asp | | |
| 945 | 950 | 955 |
| Ala His Arg Cys Asp His Cys Arg Gly Arg Asp Phe Ile Pro Tyr His | | |
| 965 | 970 | 975 |
| His Phe Thr Val Asn Ile Arg Leu Asn Ala Leu Asp Met Asp Ala Ile | | |
| 980 | 985 | 990 |
| Lys Lys Met Ala Met Ser His Gly Phe Lys Thr Glu Glu Lys Leu Ile | | |
| 995 | 1000 | 1005 |
| Leu Ser Asn Ser Glu Ile Pro Glu Thr Ser Ala Phe Phe Pro Glu Asn | | |
| 1010 | 1015 | 1020 |
| Arg Ser Pro Glu Glu Ile Arg Glu Lys Ile Leu Asn Phe Phe Asp His | | |
| 1025 | 1030 | 1035 |
| Val Leu Thr Lys Met Lys Thr Ser Asp Glu Asp Ile Ile Pro Leu Glu | | |
| 1045 | 1050 | 1055 |
| Ser Cys Gln Cys Glu Glu Ile Leu Glu Ile Val Ile Leu Pro Leu Ala | | |
| 1060 | 1065 | 1070 |
| His His Phe Leu Ala Leu Gly Glu Asn Asp Lys Ala Leu Tyr Tyr Phe | | |
| 1075 | 1080 | 1085 |
| Leu Glu Ile Ala Ser Ala Tyr Leu Ile Phe Cys Asp Asn Tyr Met Ala | | |
| 1090 | 1095 | 1100 |
| Tyr Met Tyr Leu Asn Glu Gly Gln Lys Leu Leu Lys Thr Leu Lys Lys | | |
| 1105 | 1110 | 1115 |
| Asp Lys Ser Trp Ser Gln Thr Phe Glu Ser Ala Thr Phe Tyr Ser Leu | | |
| 1125 | 1130 | 1135 |
| Lys Gly Glu Val Cys Phe Asn Met Gly Gln Ile Val Leu Ala Lys Lys | | |
| 1140 | 1145 | 1150 |
| Met Leu Arg Lys Ala Leu Lys Leu Leu Asn Arg Ile Phe Pro Tyr Asn | | |

| | | |
|---|------|-----------|
| 1155 | 1160 | 1165 |
| Leu Ile Ser Leu Phe Leu His Ile His Val Glu Lys Asn Arg His Phe | | |
| 1170 | 1175 | 1180 |
| His Tyr Val Asn Arg Gln Ala Gln Glu Ser Pro Pro Pro Gly Lys Lys | | |
| 1185 | 1190 | 1195 1200 |
| Arg Leu Ala Gln Leu Tyr Arg Gln Thr Val Cys Leu Ser Leu Leu Trp | | |
| 1205 | 1210 | 1215 |
| Arg Ile Tyr Ser Tyr Ser Tyr Leu Phe His Cys Lys Tyr Tyr Ala His | | |
| 1220 | 1225 | 1230 |
| Leu Ala Val Met Met Gln Met Asn Thr Ala Leu Glu Thr Gln Asn Cys | | |
| 1235 | 1240 | 1245 |
| Phe Gln Ile Ile Lys Ala Tyr Leu Asp Tyr Ser Leu Tyr His His Leu | | |
| 1250 | 1255 | 1260 |
| Ala Gly Tyr Lys Gly Val Trp Phe Lys Tyr Glu Val Met Ala Met Glu | | |
| 1265 | 1270 | 1275 1280 |
| His Ile Phe Asn Leu Pro Leu Lys Gly Glu Gly Ile Glu Ile Val Ala | | |
| 1285 | 1290 | 1295 |
| Tyr Val Ala Glu Thr Leu Val Phe Asn Lys Leu Ile Met Gly His Leu | | |
| 1300 | 1305 | 1310 |
| Asp Leu Ala Ile Glu Leu Gly Ser Arg Ala Leu Gln Met Trp Ala Leu | | |
| 1315 | 1320 | 1325 |
| Leu Gln Asn Pro Asn Arg His Tyr Gln Ser Leu Cys Arg Leu Ser Arg | | |
| 1330 | 1335 | 1340 |
| Cys Leu Leu Leu Asn Ser Arg Tyr Pro Gln Leu Ile Gln Val Leu Gly | | |
| 1345 | 1350 | 1355 1360 |
| Arg Leu Trp Glu Leu Ser Val Thr Gln Glu His Ile Phe Ser Lys Ala | | |
| 1365 | 1370 | 1375 |
| Phe Phe Tyr Phe Val Cys Leu Asp Ile Leu Leu Tyr Ser Gly Phe Val | | |
| 1380 | 1385 | 1390 |
| Tyr Arg Thr Phe Glu Glu Cys Leu Glu Phe Ile His Gln Tyr Glu Asn | | |
| 1395 | 1400 | 1405 |
| Asn Arg Ile Leu Lys Phe His Ser Gly Leu Leu Leu Gly Leu Tyr Ser | | |

| | | |
|---|------|-----------|
| 1410 | 1415 | 1420 |
| Ser Val Ala Ile Trp Tyr Ala Arg Leu Gln Glu Trp Asp Asn Phe Tyr | | |
| 1425 | 1430 | 1435 1440 |
| Lys Phe Ser Asn Arg Ala Lys Asn Leu Leu Pro Arg Arg Thr Met Thr | | |
| 1445 | 1450 | 1455 |
| Leu Thr Tyr Tyr Asp Gly Ile Ser Arg Tyr Met Glu Gly Gln Val Leu | | |
| 1460 | 1465 | 1470 |
| His Leu Gln Lys Gln Ile Lys Glu Gln Ser Glu Asn Ala Gln Ala Ser | | |
| 1475 | 1480 | 1485 |
| Gly Glu Glu Leu Leu Lys Asn Leu Glu Asn Leu Val Ala Gln Asn Thr | | |
| 1490 | 1495 | 1500 |
| Thr Gly Pro Val Phe Cys Pro Arg Leu Tyr His Leu Met Ala Tyr Val | | |
| 1505 | 1510 | 1515 1520 |
| Cys Ile Leu Met Gly Asp Gly Gln Lys Cys Gly Leu Phe Leu Asn Thr | | |
| 1525 | 1530 | 1535 |
| Ala Leu Arg Leu Ser Glu Thr Gln Gly Asn Ile Leu Glu Lys Cys Trp | | |
| 1540 | 1545 | 1550 |
| Leu Asn Met Asn Lys Glu Ser Trp Tyr Ser Thr Ser Glu Leu Lys Glu | | |
| 1555 | 1560 | 1565 |
| Asp Gln Trp Leu Gln Thr Ile Leu Ser Leu Pro Ser Trp Glu Lys Ile | | |
| 1570 | 1575 | 1580 |
| Val Ala Gly Arg Val Asn Ile Gln Asp Leu Gln Lys Asn Lys Phe Leu | | |
| 1585 | 1590 | 1595 1600 |
| Met Arg Ala Asn Thr Val Asp Asn His Phe | | |
| 1605 | 1610 | |

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<213> Artificial Sequence

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<223> Description of Artificial Sequence:human soluble
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<400> 12

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atcttgtctg cttggacatc ctgctttatt ctggttttgt ttatagaaca tttgaagaat 4380
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tcctgatgag agctaatacc gtggacaatc atttctaa 5018

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/29872

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C12Q 1/68 ; C07K 1/00; C12N 15/00, 5/00

US CL : 435/6, 232, 325, 320.1; 530/350, 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 232, 325, 320.1; 530/350, 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, BIOSIS, BIOTECHDS, SCISEARCH, HCAPLUS, NTIS, LIFESCI, BRS/EAST (USPAT)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------------|---|---------------------------------------|
| X ----- Y | BUCK et al., Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals, P.N.A.S. U.S.A., January 1999, Vol. 96, pages 79-84, see the entire article. | 1-14 ----- 15 |
| X ----- Y | COUDART-CAVALLI et al., Bifunctional structure of two adenylyl cyclases from myxobacterium Stigmatella aurantiaca, Biochimie, December 1997, Vol. 79, pages 757-767, see the entire article and the attached sequence alignment for less than 16% homology in the catalytic domain. | 1,5-7, 9, 14 ----- 8, 10-11, 15 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | |
|---|--|
| * Special categories of cited documents: | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" document defining the general state of the art which is not considered to be of particular relevance | "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" earlier document published on or after the international filing date | "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" document member of the same patent family |
| "O" document referring to an oral disclosure, use, exhibition or other means | |
| "P" document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

03 JANUARY 2001

Date of mailing of the international search report

22 MAR 2001

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MARYAM MONSHIPOURI

Telephone No. (703) 308-1083

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/29872

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---------------------|--|--|
| X,P ----- Y,P | Database EST, NCI-CGAhttp://www.ncbi.nlm.nih.gov/ncicgap, , No. AW592474, hf43a12.x1 Soares-NFL-T-GBC-S1 Homospiens cDNA clone IMAGE:2934622 3' similar to TR:Q9Z286 Q9Z286 Soluble Adenylyl Cyclase, 22 March 2000, see the sequence alignment. | 1-3, 5-7, 12-13 ----- 8-11, 14-15 |
| X ----- Y | ITOH et al., Automated filtration-based high-throughput plasmid preparation system, Genome Res., November 1999, Vol.9, No.5, pages 463-470, see Methods section and the attached sequence alignment. | 1, 2, 5-7, 9, 12- 13, 14 ----- 7-8, 10-11, 15 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/29872

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-15

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-15, drawn to an isolated nucleic acid molecule encoding adenylyl cyclase, vectors and host cells comprising said molecule, oligonucleotide that specifically hybridize to said molecule under stringent conditions and methods of expressing said molecule.

Group II, claim(s) 16-20 and 36-46, drawn to methods of treatment comprising administering to a whole cell or to a subject in need thereof a modulator of adenylyl cyclase in an amount sufficient to increase or decrease cyclase activity.

Group III, claim(s) 21, drawn to a method of quantifying bicarbonate in a body sample comprising contacting the body with adenylyl cyclase.

Group IV, claim(s) 22-23, drawn to a method of screening for modulators of said cyclase.

Group V, claim(s), 24-30, drawn to a method of detecting the expression of said cyclase comprising detecting the presence of said cyclase using a binding partner specific to said cyclase.

Group VI, claim(s), 31-35, drawn to a method of diagnosing a disease comprising detecting increase or decrease in the level of cyclase.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

the common special technical feature of Groups II and IV is modulators of the cyclase which is distinct from the special technical feature of GROUP III, V and VI (which is the cyclase) and that of Group I (which is the DNA). Groups II and IV however, are considered to be 2 different categories of invention and lack unity as they are not drawn to combination of categories (i.e. categories 1-5), specified in 37 CFR section 1.475(b) and thus lack unity.

Similarly, Groups III, V and VI share a common technical feature but they lack unity because they are considered to be 3 categories of invention and are not drawn to combination of categories (i.e. categories 1-5), specified in 37 CFR section 1.475(b).